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THE MUCIN OF BOVINE SUBLINGUAL GLANDS

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Abstract—Extracts of bovine sublingual glands were fractionated and the materials (mucin) responsible for the viscous stringy properties of the extracts were extensively purified. The progress of purification was followed by electrophoretic, ultracentrifugal and viscometric measurements. The purest product contained 23·3 per cent N-acetyl-hexosamine, 17·8 per cent sialic acid, 7·6 per cent 6-deoxyhexose, 20·6 per cent hexose (as galactose), and about 25 per cent protein. The molar ratios were approximately: hexosamine, 2; sialic acid, 1; 6-deoxyhexose, 1; hexose as galactose, 2. The electrophoretic mobility of the product was $-4\cdot6 \times 10^{-5}$ cm^2/sec/V at pH 7·4, ionic strength 0·1. The sedimentation constant was 8·6 S in the same buffer.

The composition of the mucin clot was also investigated and found to be a dissociable mucoprotein. The mucin appears to act as one of the anionic components in the formation of the mucin clot. The purest mucin itself gave no clot by adjustment of the pH to 3·5 except in the presence of added serum albumin.

INTRODUCTION

THE nature and composition of the mucin components in human saliva responsible for its viscous stringy characteristics have not been established. Human saliva shows rapid alterations in its properties, and the individual secretions are difficult to obtain in sufficient quantities for chemical isolations. Hence, most of the previous studies on salivary mucins have been made on extracts of animal salivary glands, most often bovine submaxillary glands. In previous work, submaxillary mucin has been isolated as the mucin clot (HAMMARSTEN, 1888), but the products were not well characterized and probably had reduced solubility characteristics. Other studies generally were of products obtained from the clot (BLIX, 1936; HEIMER and MEYER, 1956) or directly from the extract (McCREA, 1953; ODIN, 1958) by drastic chemical procedures so that their relationship to the original extracts is not clear. Only one prior study of sublingual mucin has been reported, and degradative procedures were employed in this (TANABE, 1938, 1939).

In the present work, a method has been developed which separates from bovine sublingual glands the principal "mucin" responsible for the rheological characteristics of the extracts. Although the purified products were not completely homogeneous, the mucin appears to belong to the class of glycoproteins as defined by PIGMAN, NISIZAWA and TSUIKI (1959). This paper also describes the composition and nature of the mucin clot, which appears to be a salt-like complex (mucoprotein) formed from the mucin(s) and protein(s).

EXPERIMENTAL

Materials

The bovine sublingual glands were obtained at a slaughter-house and kept frozen until used. They were obtained from the J. M. Thomason Packing Company, Bessemer, Alabama, through the courtesy of Dr. H. E. HARRIMAN. Each sublingual gland consisted of distinct dorsal and ventral portions. The dorsal part was long, thin, and pale yellow in colour. It consisted of a band of numerous small individual glands, which lay beneath the mucous membrane of the floor of the mouth. Each of the small individual glands emptied into the oral cavity by a separate duct. The ventral part, salmon pink in colour, was shorter and thicker and lay ventral to the anterior portion of the dorsal part. The ventral part of one typical gland weighed nearly 6 g. The weight of the dorsal part could not be measured accurately because of the difficulty of isolating all of the small glands but probably weighed about 2–3 g.

Samples of the glands were examined by Drs. C. KLAPPER and T. WEATHERFORD. Two samples were taken from the dorsal and three from the ventral portion. Histological sections, prepared from tissue fixed in Bouin's solution, were stained with haematoxylin and eosin. In all of the samples, most of the secreting alveoli were of the mucous type. However, some were of a mixed type, consisting of a mucous alveolus with a demilune of serous cells.

The connective tissue septa were well developed, but the gland did not have a distinct capsule. As a result, the glandular tissue was contaminated with surrounding connective tissue and blood. The first extracts had a red colour.

In one preliminary experiment, the two parts were extracted separately. Viscous extracts and mucin clots were obtained in both cases. In most of the work, however, the glandular material included both parts of the gland.

Several batches of commercial material appeared to be incorrectly identified by the suppliers and seemed to be large lymph nodes.

Analytical methods

Hexosamine. Hexosamine was determined by the method of Elson and Morgan as modified by BOAS (1953). The materials were hydrolysed with 4 N hydrochloric acid at 100°C for 7 hr and the acid was removed by evaporation *in vacuo* over solid sodium hydroxide. Glucosamine hydrochloride was used as the standard.

Sialic acid. This was determined by a modification of the direct Ehrlich method (PIGMAN, HAWKINS, BLAIR and HOLLEY, 1958). N-acetylneuraminic acid was used as the standard.

6-Deoxyhexose. This was determined by the method of DISCHE and SHETTLES (1948) using L-fucose as the standard. Since the interference by hexoses was negligible in their procedure CyR 10, this procedure was used throughout this work.

Hexose. A modified anthrone method (HANSON, SCHWARTZ and BARKER, 1955) was used. Under these conditions, L-fucose also produced a considerable amount of chromogen, whereas the optical density of galactose was only half of that for equivalent glucose. To obtain hexose values, the optical densities were first corrected for the amount of 6-deoxyhexose and then were calculated as both glucose and galactose.

Hexuronic acid. Several fractions were analysed for hexuronic acid by the carbazole method of DISCHE (1955). Glucurone served as the standard. The values are not very accurate because of the relatively low concentration of hexuronic acid and the high concentration of hexose in the products.

Protein. Protein contents were determined by the biuret (ROSENTHAL and CUNDIFF, 1956) and phenol (LOWRY, ROSENBROUGH, FARR and RANDALL, 1951) methods. For most of the fractions, both methods gave similar results. Crystalline bovine serum albumin was used as the standard.

Physical measurements

Electrophoresis. The electrophoretic analyses were conducted at $1.0°C$ in a Perkin–Elmer model 38 Tiselius-type apparatus with 6 ml cell. The ascending patterns were used for all mobility calculations. In this work, all the fractions were prepared as solutions in the diluted buffer (see below). For electrophoretic or other physical analyses, the solutions were dialysed against the appropriate buffers. Buffers used were phosphate buffers of ionic strength 0.1 at pH 7.4 (0.0033 M NaH_2PO_4–0.0167 M Na_2HPO_4–0.0455 M NaCl) and at pH 10.8 (0.0051 M Na_2HPO_4–0.0069 M Na_3PO_4–0.0433 M NaCl).

Ultracentrifugation. The analyses were carried out in the Spinco Model E analytical ultracentrifuge at 59,780 r.p.m. (259,700 g), using the 12 mm standard analytical cell. Mr. E. GRAMLING made these measurements.

Viscometry. Relative viscosities were measured in phosphate buffer, pH 7.4 and ionic strength 0.1, at $30.0°C$. A Cannon–Manning semimicro viscometer was used (No. 200). The buffer (0.2 ml) had a flow time of 9.8 sec.

Methods of preparation

A Clark and Lubs' phosphate buffer, pH 7, which had been diluted 10 times with water, was used as extractant. This "diluted buffer" also served as the solvent for products throughout the process of fractionation. All the steps of extraction and fractionation were carried out at $2-4°C$.

Extraction. The frozen glands were thawed at room temperature, cut into small pieces (ca. 10 mm cubes) and freed as completely as possible of connective and fatty tissues. In one preliminary experiment, these small pieces were extracted directly (Method 1). Fourteen changes of 100 ml of the diluted buffer were used for the extraction of 66 g of the trimmed glands. The first nine extractions were carried out at $5°C$ with occasional shaking. The time of each extraction was 14 hr with the exception of the first, when the time of extraction was 3 hr. At the tenth extraction, the glands and buffer were placed in a refrigerator at $2°C$, and the time was increased to 48 hr. The four successive extractions were carried out in this manner. The analyses of these extracts are shown in Figs. 1 and 2.

Most extractions, however, were made by another procedure (Method 2). In a typical experiment, 340 g of trimmed glands were passed through a meat grinder, and these ground glands were stirred mechanically with three changes (1000 ml, 1000 ml and 700 ml) of the diluted buffer. The time of each extraction was 24 hr.

The extracts were separated from the residues by centrifugation at 20,000 r.p.m. (26,360 g) for 30 min.

Preparation of mucin clot. To prepare mucin clots, the various extracts were cooled to 0°C in an ice–water bath, and 0·1 N hydrochloric acid was added very slowly with vigorous stirring until the pH of the solution reached 3·5. The resulting mucin clot was immediately suspended in cold water, and enough 0·1 N sodium hydroxide was added with stirring to bring the pH to 7. A clear solution was obtained. Mucin was reprecipitated and redissolved as described above and finally dialysed against the diluted buffer. The supernatant of the clot was neutralized with sodium hydroxide and again brought to pH 3·5. Any precipitate or turbidity which appeared was removed by centrifugation, and the clear supernatant was dialysed against the buffer.

Fractionation of extracts with ammonium sulphate. A solution of ammonium sulphate saturated at 2–4°C was added to an extract under constant stirring, so as to bring the concentration of ammonium sulphate slowly to 10 per cent saturation. The solution was allowed to stand for 12 hr in a refrigerator, and the precipitate was collected by centrifugation at 20,000 r.p.m. (26,360 g) for 30 min. The precipitate was suspended in a small volume of the diluted buffer and dialysed against several changes of the same buffer.

The supernatant was submitted to further fractionation with ammonium sulphate, and the precipitates at 20, 30, 40, 50, 60, 70 and 80 per cent saturation (at 2–4°C) were obtained in the same manner. The precipitate at 20 per cent saturation usually was gel-like, and, after separation of this precipitate, the solution was no longer viscous.

The supernatant from 80 per cent saturation was finally brought to full saturation by adding crystalline ammonium sulphate. A small flocculent precipitate appeared, which was centrifuged off, dissolved in the buffer and dialysed. A portion of this final supernatant was dialysed first against running water, then against a 25 per cent aqueous solution of polyvinylpyrrolidone and finally against the diluted buffer.

The fractions from the pooled extracts (2000 ml) from 340 g of ground glands obtained in the above fashion were analysed for protein, sialic acid, hexose, 6-deoxy-hexose and hexuronic acid. The results are given in Table 2.

Purification of the fraction precipitated at 10–20 per cent saturation of ammonium sulphate. The fraction precipitated at 10 to 20 per cent saturation of ammonium sulphate (Product AS20) was further fractionated. A portion of this fraction was reprecipitated three times from the diluted buffer at 20 per cent saturation of ammonium sulphate (Product AS20b).

Another portion was reprecipitated once with 20 per cent saturated ammonium sulphate (Product AS20a). Product AS20a was then subjected to fractionation with increasing amounts of sodium sulphate. In one experiment, 2·5 ml of a 25 per cent aqueous solution of sodium sulphate was added to 25 ml of the fraction (AS20a) with continuous stirring, and a very small precipitate was centrifuged off. The addition of 6·3 ml of a 25 per cent sodium sulphate solution to the supernatant did not cause any change in appearance. However, when the mixture was kept 18 hr at 2–4°C and centri-

fuged at 26,000 r.p.m. (44,590 g) for 45 min, a voluminous, transparent gel was deposited. The gel was agitated with a small volume of water and the mixture was dialysed against the diluted buffer. The fraction obtained was reprecipitated again under the same conditions (Product AS20c). The composition of these fractions is presented in Table 3.

RESULTS

Extraction and preparation of mucin clot. A preliminary experiment (Method 1) was carried out in order to study the course of extraction of sialic acid-containing material from bovine sublingual glands. Fig. 1 shows the quantities of sialic acid in
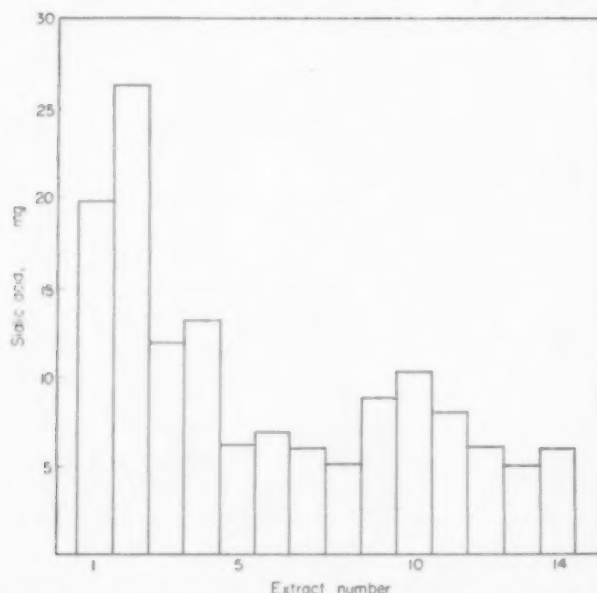
FIG. 1. The amounts of sialic acid in successive extracts (Method 1).

successive extracts. Although more than half of the total sialic acid was found in the first four extracts, the successive extracts gave materials which were progressively richer in sialic acid (Fig. 2). Practically all of the extracts gave a mucin clot, and some purification was brought about by mucin clot formation (Fig. 2). However, the composition of the precipitated mucin clot was not constant for the several extracts (Fig. 2).

When the ground glands were extracted with the aid of mechanical stirring (Method 2), the rate of extraction of the sialic acid materials was much increased. By three extractions over a period of 64 hr, approximately 830 mg of combined sialic acid was extracted from 340 g of moist glands. The ratio of sialic acid to protein of this pooled extract was 0·074 and was higher than any of the first six extracts prepared by Method 1 (Fig. 2).
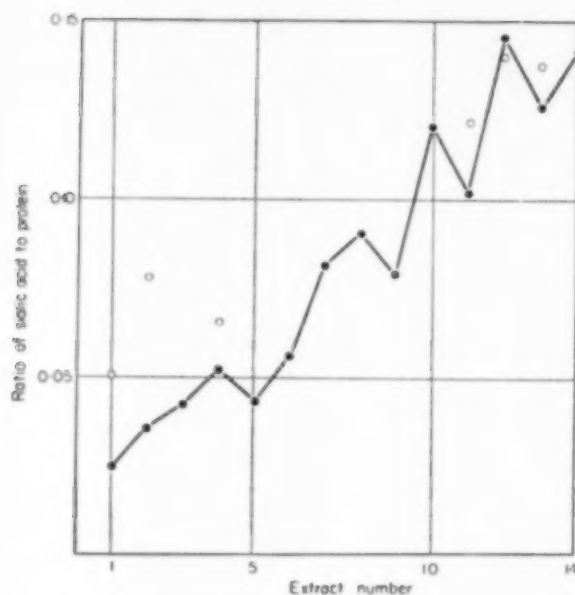
Fig. 2. Ratio of sialic acid to protein for successive extracts and mucin clots (Method 1). ●, Extract; ○, Mucin clot.

An 80 ml portion of the pooled extract made by Method 2 was fractionated into mucin clot and supernatant as described above. Table 1 shows the distribution of several components of these two fractions. The original extract contained a small amount of hexuronic acid, most of which appeared in the precipitated mucin clot. Qualitatively, the mucin clot contained no ester–sulphate after tests with barium

TABLE 1.   COMPOSITION OF MUCIN CLOT AND SUPERNATANT FROM
THE EXTRACT OF THE SUBLINGUAL GLANDS

Fraction	Protein* (mg)		Sialic acid† (mg)	6-Deoxyhexose‡ (mg)	Hexuronic acid§ (mg)
	Biuret	Phenol			
Extract ‖	404	438	30·0	8·6	0·52
Mucin clot	134	105	11·0	3·4	0·40
Supernatant	192	225	9·2	4·0	0·03

* Calculated as bovine serum albumin.
† Expressed as N-acetylneuraminic acid.
‡ As L-fucose.
§ As glucurone.
‖ Composition of the extract used for this fractionation. The loss of the material in the fractionation probably was due to the purification of the derived fractions (see text).

chloride on the hydrolysate produced by 1 N hydrochloric acid for 10 hr at 100°C. The mucin clot showed no maximum at 260 mμ in ultra-violet absorption spectrum and thus appeared free of nucleic acids. A comparison of the viscosity of the clot and extract indicated that most of the material responsible for the viscosity of the extract was associated with the mucin clot (Fig. 5).

Fractionation of extracts with ammonium sulphate. Table 2 shows the results of an analysis of the fractions obtained by fractionation with increasing concentrations of ammonium sulphate. Two peaks of distribution of sialic acid were evident among these fractions. The ratio of sialic acid to protein (biuret method) for the fraction precipitated at 10–20 per cent saturation of ammonium sulphate (AS20) was 0·135 and higher than that for any other fraction from the same original extract including the mucin clot. This fraction yielded a very viscous solution, and upon acidification the solution gave a typical mucin clot. The solution of the fraction precipitated at 60–70 per cent saturation (Product AS70) was not very viscous. When acidified, it gave a flocculent precipitate. The fraction precipitated at 40–50 per cent saturation had the highest ratio of sialic acid to the other sugar components. The total amount of sialic acid recovered from these ten fractions corresponded to 78 per cent of that in the original extract. Only 33 per cent appeared in AS20.

TABLE 2.   COMPOSITION OF THE FRACTIONS PRECIPITATED FROM THE SUBLINGUAL
EXTRACT BY INCREASING CONCENTRATIONS OF AMMONIUM SULPHATE*

Percentage saturation of ammonium sulphate at 2–4°C	Protein† (mg)		Sialic acid‡ (mg)	Hexose§ (mg)		6-Deoxy-hexose‖ (mg)	Hexuronic acid¶ (mg)
	Biuret	Phenol		As glucose	As galactose		
0–10	223	162	14	10	19	8	
10–20	1412	1404	189	132	250	71	0·86
20–30	651	672	28	16	31	9	0·63
30–40	559	559	41	23	43	16	0·65
40–50	1043	1139	70	31	57	22	1·04
50–60	832	846	74	50	92	23	1·14
60–70	1830	1826	128	95	180	47	2·09
70–80	982	903	37				
80–100	Trace		Trace				
Supernatant	Trace		Trace				

* Extract originally contained 750 mg of sialic acid and 215 mg of 6-deoxyhexose.
† Calculated as bovine serum albumin.
‡ Expressed as N-acetylneuraminic acid.
§ Corrected for L-fucose.
‖ Expressed as L-fucose.
¶ Expressed as glucurone.

Fractionation of the mucin clot with ammonium sulphate. In order to elucidate the relationship of the mucin clot to the fraction AS20, a portion of the mucin clot (prepared from another batch of the gland), which contained 13 mg of sialic acid,

was fractionated with ammonium sulphate essentially in the same manner as described above. Fractions rich in sialic acid were obtained both at 20 (sialic acid, 6·5 mg) and at 70 per cent saturation (6·1 mg). After removal of ammonium sulphate, the fraction precipitated at 20 per cent saturation was not soluble in the diluted buffer. Although the fraction gave a homogeneous and viscous solution, centrifugation at 20,000 r.p.m. (26,360 g) for 30 min yielded a gel-like precipitate. The viscosity of the supernatant decreased considerably and the solution was turbid. As was found also in the preliminary studies, the mucin seemed to denature in the formation of the mucin clot.

Further purification of the fraction precipitated at 10–20 *per cent saturation of ammonium sulphate.* Table 3 gives the composition of the products derived from AS20 by several purification procedures. As seen from the table, much of the protein originally present in AS20 was removed by repeating the ammonium sulphate precipitation or by employing a sodium sulphate fractionation. Sodium sulphate seemed to be better than ammonium sulphate as a fractionation agent. The purest product (AS20c) was prepared by this procedure. Neither nucleic acid nor hexuronic acid was detected in any of these purified sub-fractions.

Electrophoresis. Electrophoretic examination of the products at various stages of purification was made at pH 7·4 and 10·8. As seen from Fig. 3, even the purest
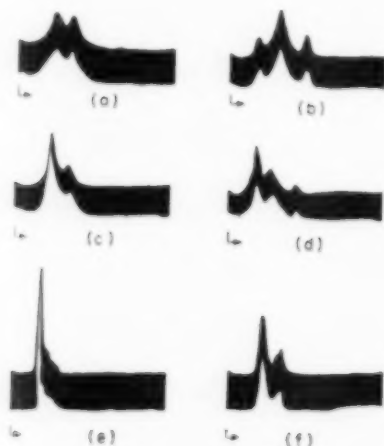
Fig. 3. Electrophoretic patterns (ascending) of the original extract, product AS20 and AS20c at 120 min. The solutions contained 0·3–0·4 mg of sialic acid per ml. (a) Original extract at pH 7·4, mobilities are −5·9 and −4·4 × 10⁻⁵ cm²/sec/V; (b) at pH 10·8, mobilities are −9·8, −6·8 and −4·1; (c) AS20 at pH 7·4, mobilities are −6·4 and −4·5; (d) at pH 10·8, mobilities are −9·3, −6·5 and −4·5; (e) AS20c at pH 7·4, mobility of slower boundary is −4·6; (f) at pH 10·8, mobilities are −7·5, −6·9 and −4·9.

product (AS20c) was not completely homogeneous at both pH values. It is also evident from the figure that, with the progress of purification, the boundary with a mobility of about $-4·6 \times 10^{-5}$ cm²/sec/V (pH 7·4) increased relatively and sharpened.

TABLE 3. COMPOSITION OF SUB-FRACTIONS DERIVED FROM THE FRACTION PRECIPITATED AT 10–20 PER CENT SATURATION OF AMMONIUM SULPHATE (PRODUCT AS20) BY SEVERAL PURIFICATION PROCEDURES*

Fraction	Protein† (mg)	Sialic acid‡ (mg)	Hexose§ (mg)	6-Deoxy-hexose‖ (mg)
Precipitated at 10–20 per cent saturation of ammonium sulphate (AS20)	259·3	35·0	33·3	13·3
Reprecipitated with ammonium sulphate (once) (AS20a)	97·0	22·3	21·2	10·0
Reprecipitated with ammonium sulphate three times (AS20b)	31·5	14·2	13·9	—
Precipitated twice with sodium sulphate after one reprecipitation with ammonium sulphate (AS20c)	30·4	17·0	18·5	8·8

* All the fractions were derived from the same amount of AS20, and the composition of AS20 used for each purification is listed at the top of the table.
† By phenol method, calculated as bovine serum albumin.
‡ Expressed as N-acetylneuraminic acid.
§ By anthrone method as glucose, not corrected for L-fucose.
‖ Expressed as L-fucose.

Ultracentrifugation. During sedimentation at 259,700 g, the fraction AS20b separated into three components. No significant differences could be observed between the patterns at pH 7·4 and 10·8. Typical patterns and sedimentation constants are given in Fig. 4. A comparison with patterns of the extract indicated that the very sharp peak of 8·6 S probably represented the viscous component.

FIG. 4. Ultracentrifugal patterns of original extract and of AS20b at 36 min. The concentration of sialic acid in the solutions was 0·3–0·4 mg/ml. (a) Original extract at pH 7·4; (b) AS20b at pH 7·4, $S_{20,w}$ are 10·8, 8·6 and 4·2; (c) at pH 10·8, $S_{20,w}$ are 11·2, 8·6 and 4·1.

Viscometry. The relative viscosity values for solutions in phosphate buffer, pH 7·4, ionic strength 0·1, are shown in Fig. 5. Since the main purification was the removal of protein of low viscosity, the relative viscosity based on dry weight would be expected to be increased. If the sialic acid is a part of the viscous component, the viscosity should be related to its concentration. When the viscosities were compared

on a sialic acid basis, product AS20 showed much higher viscosities than those for the original extract. This result indicates that, despite the distribution of sialic acid into the several fractions, a compound containing sialic acid is responsible for the high viscosity of this fraction (AS20) and also of the original extract. On the other
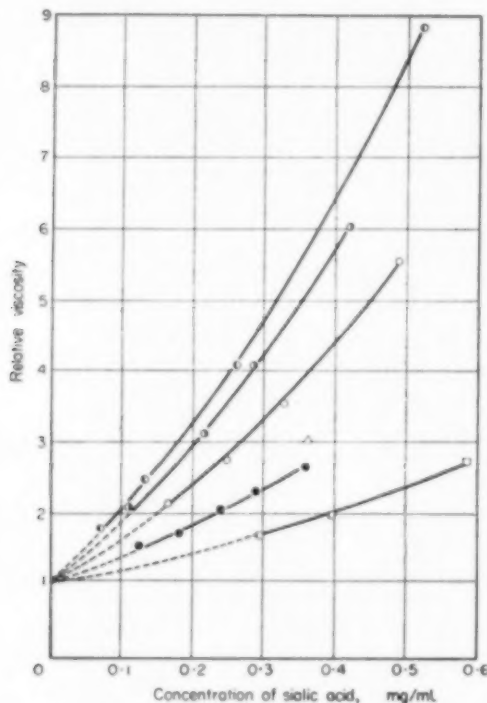
FIG. 5. Relative viscosities of the various products in phosphate buffer, pH 7·4, ionic strength 0·1, referred to the concentration of sialic acid. ●, extract; ○, AS20; □, AS70; ◑, AS20b; ◐, AS20c; △, Mucin clot.

hand, the sialic acid contained in the fraction AS70 does not seem to be related to the viscous nature of the original extract. Further purification of AS20 also caused an additional increase in viscosity. This increase suggested that no degradation occurred in the course of purification.

The effect of velocity gradient was also studied by measuring the viscosity of the product AS20c in three viscometers of widely different rates of flow. However, the measured viscosities were only slightly dependent on the flow rate as shown in Table 4.

Ability to form a mucin clot. The fractions AS20 and AS70 were examined for their ability to form a mucin clot. Prior to the test, both fractions were diluted with the diluted buffer to give solutions containing 0·3 mg of sialic acid per ml. Product

TABLE 4.   RELATIVE VISCOSITIES AT 30·0°C OF THE PRODUCT AS20c
IN PHOSPHATE BUFFER OF pH 7·4, μ 0·1 IN
VISCOMETERS OF DIFFERENT FLOW RATES

Concentration of sialic acid (mg/ml)	Viscosity in Cannon–Manning viscometer number		
	100	200	300
0·520	10·31	8·79	8·08
0·260	5·02	4·09	3·92

AS70 initially gave a turbidity which changed to a flocculent precipitate after several minutes. Only 32 per cent of the sialic acid was precipitated. In contrast, AS20 immediately formed a typical clot, in which 85 per cent of the sialic acid originally present was found. When AS20c was brought to pH 3·5 under the same condition, no clot appeared. Centrifugation at 20,000 r.p.m. (26,360 g) for 30 min produced a small amount of precipitate, and the supernatant remained viscous. However, when a small amount of bovine serum albumin was added to the solution of AS20c, adjustment of the pH to 3·5 gave a typical clot.

DISCUSSION

Extracts of bovine sublingual glands have been fractionated, and the material responsible for the viscous stringy properties of the extracts has been purified considerably. The purest material contained 23·3 per cent hexosamine (as N-acetylglucosamine), 17·8 per cent sialic acid (as sodium N,O-diacetyl-neuraminate), and 7·6 per cent 6-deoxyhexose (as L-fucose). The hexose content was about 20·6 per cent, when calculated as galactose. The percentage composition and molar ratios of these components are shown in Table 5 for fractions at different stages of the purification.

The most highly purified material (AS20c) exhibited a molar ratio of the carbohydrate constituents of approximately: hexosamine, 2; sialic acid, 1; apparent L-fucose, 1; apparent D-galactose, 2. Products of a lower degree of purification and the mucin clot, although containing much more protein, had similar ratios. The actual identification of these constituents will require additional work, but this choice is very likely because of the identification of N,O-diacetylneuraminic acid (BLIX, LINDBERG, ODIN and WERNER, 1956), D-galactose and L-fucose (BERGGÅRD and WERNER, 1958) in similar products. The hexosamine would be expected to be preponderantly N-acetylglucosamine since the mucin seems to belong to the class of "fucomucins" (ODIN, 1958).

The purified sublingual mucins differ markedly in their chemical composition from the submaxillary products in which the principal carbohydrates are N-acetylgalactosamine and sialic acid in equimolar amounts (NISIZAWA and PIGMAN, 1959). The relatively low concentration of sialic acid in sublingual mucin is probably

responsible for its lower electrophoretic mobility (-4.6×10^{-5} cm^2/sec/V) compared to submaxillary mucin under the same conditions (NISIZAWA and PIGMAN, 1959).

The progress of purification was followed by electrophoretic, ultracentrifugal and viscometric measurements. The results of the viscometric measurements, especially, indicate strongly that the product obtained (AS20c) here is the principal viscous component in sublingual extract. As even the purest product was not homogeneous, the significance of the protein portion, present to the extent of 25 per cent, is still not clear. This protein may be only a contaminant or it may make up an essential part of the mucin, as a glycoprotein or mucoprotein.

TABLE 5. CHEMICAL COMPOSITION OF THE SEVERAL PRODUCTS FROM
BOVINE SUBLINGUAL EXTRACT

	As percent (dry wt.)			As molar ratio		
	Mucin clot	AS20	AS20c	Mucin clot	AS20	AS20c
Hexosamine as N-acetylglucosamine	8·0	12·1	23·3	1·00	1·00	1·00
Sialic acid as sodium N,O-diacetylneura-minate	8·7	10·3	17·8	0·64	0·49	0·45
6-deoxyhexose as L-fucose	2·2	3·2	7·6	0·36	0·35	0·44
Hexose*						
as glucose	4·3	6·0	11·2	0·67	0·60	0·59
as galactose	8·0	11·3	20·6	1·22	1·15	1·08
Protein†	69·0	63·0	26·5	—	—	—

* Corrected for 6-deoxyhexose.
† By phenol method, calculated as bovine serum albumin.

The comparison of the chemical composition with the viscosity of the products at various stages of purification indicates that some of the combined sialic acid is not related to the viscous component (Tables 3 and 5, and Fig. 5). The same kind of evidence was also provided from the results of fractionation of the extract with ammonium sulphate (Table 2 and Fig. 5). Two or more compounds in sublingual extract thus contain sialic acid in appreciable amounts.

BLIX, SVENNERHOLM and WERNER (1952) have stated that, for the extraction of submaxillary mucin, glands should not be minced finely as this treatment brought about co-extraction of cellular protein. However, for sublingual mucin, mincing seems desirable, and Method 2 has given better results than Method 1. The first step in the preparation of the mucin has been carefully investigated, and a comparison between the mucin clot and the fraction AS20 has been made in many respects. The results suggest strongly that the precipitation of the mucin with ammonium sulphate

is milder and more specific for the mucin than the formation of a mucin clot. Furthermore, formation of the clot denatures the mucin.

PIGMAN, GRAMLING, PLATT and HOLLEY (1959) recently established that synovial mucin is a dissociable mucoprotein formed from hyaluronic acid and albumin. In order to elucidate the nature of the clot from the sublingual gland, the products at different stages of fractionation have been examined for their ability to form a mucin clot. The fraction AS20 could be precipitated nearly quantitatively as a mucin clot, whereas AS20c lost this ability completely. On the basis of the mild nature of the fractionation procedure, and of the viscosity data, the possibility of a breakdown of a definite compound can be excluded. Since the addition of bovine serum albumin can restore this property, the situation is explained by the removal of the protein fraction. This observation indicates that the sublingual mucin clot is also a salt-like complex (mucoprotein) consisting of glycoprotein(s) (or polysaccharide) of acidic nature and of cationic protein(s) under weakly acidic conditions. The clot formed from submaxillary mucin has an analogous composition (NISIZAWA and PIGMAN, 1959). In contrast to the synovial (PIGMAN, GRAMLING, PLATT and HOLLEY, 1959) and submaxillary mucin clots (NISIZAWA and PIGMAN, 1959), the composition of the sublingual mucin clot changed for several successive extracts, especially when Method I was employed (Fig. 2). Such a situation could arise if more than one component acted as an anion in this interaction system. This explanation seems likely, as the mucin clot gave two fractions containing sialic acid after fractionation with ammonium sulphate. In addition, in agreement with the previous work (TANABE, 1938), a small amount of hexuronic acid was found in the mucin clot. The very low concentration of this component, however, suggests that it might have originated from the connective tissue surrounding the glands.

In this situation, the mucin clot itself cannot be considered as the principal viscous component of the extract. Certainly the word "mucin" should not be synonymous with "mucin clot" which appears to be a combination of several different components existing only in acid solutions. The principal viscous component, which appears to be a water-soluble glycoprotein and which is one of the anionic components of the mucin clot, is to be considered the mucin.

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STUDIES ON THE PHYSICAL PROPERTIES OF FLUOROSED ENAMEL—I

MICRORADIOGRAPHIC STUDIES

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Abstract—Thirteen permanent fluorosed teeth collected from areas where the water supply contains 2·2–5·0 p.p.m. fluoride were studied microradiographically. Most of these teeth showed areas of radiolucency. In all cases the outer layer was well calcified, as was the enamel towards the dentine. The spread of the radiolucent areas varied, whereas the depth was predictable. Where the drinking water contained less than 5·0 p.p.m. of fluoride only the subsurface outer third of the enamel was radiolucent. Teeth from Post, Texas (5·0 p.p.m. fluoride in drinking water) showed radiolucent areas extending through one-half to three-quarters of the enamel thickness. Within the affected enamel, the interprismatic substance and areas along the edge of each incremental layer appeared to be the most radiolucent.

ALTHOUGH a considerable amount of research has been directed toward elucidating various chemical differences between mottled and sound enamel, it was only recently that these differences have been related to the morphology of the tooth (JENKINS and SPIERS, 1953; BRUDEVOLD, GARDNER and SMITH, 1956; ISAAC, BRUDEVOLD, SMITH and GARDNER, 1958). There still remain many points concerning mottled enamel which need further investigation. Very little appears in the literature on the physical properties of fluorosed enamel and mechanisms responsible for the mottling are still not clear.

Most histologists (BLACK and McKAY, 1916; WILLIAMS, 1923; AINSWORTH, 1933) studying mottled enamel agree that it shows a higher degree of pigmentation than normal enamel and that this pigmentation is usually limited to the outer third. An increased permeability to dyes, a higher fluorescence in ultra-violet light and an increased radiolucency have also been described (WILLIAMS, 1923; BHUSSRY, 1956; APPLEBAUM, 1936). Lately superior methods of microradiography have been developed using fine-grained photographic emulsions which make it possible to study microstructures in teeth not revealed by previous roentgenographic techniques. Because of the suitability of microradiography for assessing mineralization of fluorosed enamel, this investigation was undertaken.

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MATERIAL AND METHODS

Thirteen permanent teeth were obtained from Clovis, New Mexico; Colorado Springs, Colorado; Doland, South Dakota; and Post, Texas, where the water supply contains 2·2, 2·7, 3·6 and 5·0 p.p.m. fluoride, respectively. Only teeth from persons who had lived continuously in these locations were included.

The teeth were sectioned and then ground and polished by the authors' modification of a technique described by HAMMARLUND-ESSLER (1955). Sections falling in the range of 60–120 μ thickness were considered suitable for microradiography. The specimen was placed in close contact with the emulsion of a 3×1 in. Kodak Spectrographic Plate Type 649-0 by wrapping a single sheet of thin cellophane around both section and plate and applying rubber elastics around the cellophane close to the section. To prevent exposure to light the whole assembly was further enclosed in black paper. This procedure was carried out in a dark room under a safelight (Kodak Wratten Series 6B).

Roentgen-ray exposures were made of the section at a target distance of 11 cm with a low voltage machine equipped with a General Electric diffraction tube with a copper target. The machine was operated at 20 kVp and a 10 mA tube current. The emerging radiation was predominantly the $K\alpha$ characteristic radiation for copper with a wavelength of 1·54 Å.

Following exposure the plates were developed in Kodak Developer D-19, fixed in Kodak Acid Fixer and washed. After drying the plates were examined microscopically.

RESULTS

The macroscopic and microradiographic appearances of thirteen teeth from areas containing different concentrations of fluoride in the drinking water are listed in Table 1. With one exception all teeth studied showed macroscopic evidence of mottling in the form of opaque white, white-yellow and brown areas. In some instances, the mottling was limited to isolated patches, in other cases there were diffuse brown areas spread over the entire enamel surface. However, the degree of mottling did not correspond entirely to the level of fluoride in the water supply. This might be expected in view of the findings of individual variations in the degree of mottling in endemic fluoride areas (DEAN and ARNOLD, 1943).

Microradiographically a large number of these teeth showed radiolucency. In all cases, the radiolucent areas were limited to the subsurface, and a radiopaque surface layer could be observed. Three classifications have been used to denote the distribution of the radiolucent areas.

(a) *Restricted*—when only a limited area of the enamel is involved.

(b) *Medium*—if a considerable amount of the buccal or lingual enamel is involved.

(c) *Extensive*—characterized by widespread radiolucent areas on buccal, lingual and occlusal enamel.

The spread of the radiolucent areas varied considerably between the three classifications and could in no way be related to the fluoride level in the water supply.

However, the depth of the radiolucent areas appeared to be directly governed by the concentration of fluoride in the drinking water. At lower fluoride levels these areas were all found in the outer third of the enamel but at the higher level (5·0 p.p.m.), the depth of the radiolucent zones was increased so that half and even three-quarters of the enamel thickness was involved.

TABLE 1.   MACROSCOPIC AND MICRORADIOGRAPHIC APPEARANCE OF TEETH
FROM AREAS WITH DIFFERENT FLUORIDE LEVELS IN WATER SUPPLY

Area	F (p.p.m.) water	Age	Macroscopic appearance	Microradiographic appearance	
				Subsurface radiolucency	Micro-structure
Clovis, N. Mex.	2·2	20–29	Limited brown	None	None
		20–29	Diffuse brown	Extensive; outer $\frac{1}{3}$	Striae Retzius
Colorado Springs	2·7	Misc.	Diffuse brown	Medium; outer $\frac{1}{3}$	Striae Retzius
			Brown; grey	Restricted; outer $\frac{1}{3}$	Striae Retzius
			Limited brown bucc.	Medium; outer $\frac{1}{3}$	Striae Retzius
			Diffuse opaque brown	Extensive; outer $\frac{1}{3}$	Striae Retzius interprism.
Doland, S. Dakota	3·6	Over 50	Limited opaque white	None	None
		20–29	Normal	None	None
		20–29	Diffuse white yellow	Medium; outer $\frac{1}{3}$	Striae Retzius
Post, Texas	5·0	20–29	Diffuse brown	Extensive; outer $\frac{3}{4}$	Striae Retzius interprism.
			Diffuse brown	Restricted; outer $\frac{1}{2}$	Striae Retzius interprism.
			Diffuse brown	Restricted; outer $\frac{1}{2}$	None
			Diffuse brown	Extensive; outer $\frac{3}{4}$	None

Fig. 1 shows a microradiograph of a tooth from Colorado Springs (2·7 p.p.m. fluoride in drinking water); an increased radiolucency of the outer third of the labial enamel can be observed. However, the radiolucency does not include the surface layer which appears to be well calcified. The striae of Retzius have been rendered visible running in an upward and outward direction. The line in the upper left field and the horizontal lines are artifacts due to the polishing procedure. However, fine radiolucent lines running at an angle to the striae can be seen. It is assumed that these lines, seen in microradiographs of longitudinal sections, are due to an increased radiolucency of the interprismatic material. This is supported by the findings of DARLING and BROOKS (1959) who reported a hypocalcification of the interprismatic substance of mottled enamel in microradiographs of transverse sections.

Microdensity tracings (Fig. 2) of X-rays of fluorosed enamel have confirmed visual observations, namely, a peak in radiopacity at the enamel surface immediately followed by a definite decrease in radiopacity of the outer third or more of the enamel.
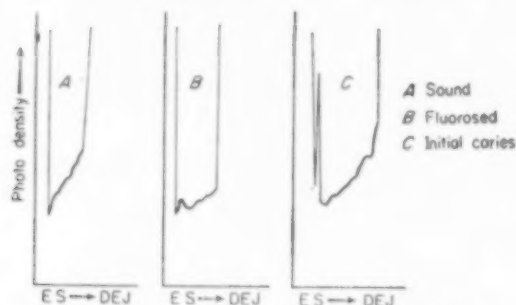
FIG. 2. Microdensity tracings of roentgenographs, comparing sound and fluorosed enamel with initial caries. ES = enamel surface; DEJ = dentino–enamel junction.

DISCUSSION

There can be little doubt that high concentrations of fluoride in the water supply ingested during the period of tooth development can interfere in both enamel matrix formation and calcification. Hypoplastic pits and surface irregularities are visible evidence of disturbances in matrix formation; however, hypocalcification cannot be detected as readily. Early investigators (MONTELIUS, MCINTOSH and MA, 1933), using chemical analyses, could find no difference in the percentage ash weight of calcium and phosphorus in normal and fluorosed enamel. However, if the variation from normal in calcium and phosphorus content is small, it may not be enough to determine the amounts present in whole enamel. Possibly different layers of the enamel should be analysed. More recently, BHUSSRY (1956) has ascribed a higher nitrogen content to mottled than to sound enamel, but was unable to correlate this with his density determinations. Radiographic studies by APPLEBAUM (1936) and our own studies prove conclusively that mottled enamel is indeed hypocalcified, in that radiolucent areas may be demonstrated which are not found in normal enamel.

Numerous animal experiments *in vivo* and with tooth cultures *in vitro* (BETHKE, KICK, HILL and CHASE, 1933; SCHOUR and SMITH, 1935; GRINSTEIN, 1942; PINDBORG, 1950, FLEMING, 1953; FLEMING and GREENFIELD, 1954) have demonstrated morphological changes in the enamel organ following prolonged administration of fluorides. These changes are manifested as alterations of the cell structure of the ameloblasts, such as appearance of vacuoles in the cytoplasm, thickening of the cell walls, loss of polarity of the nuclei and disappearance of the Golgi apparatus. Hyperaemia and disruption of pulpal blood-vessels have also been observed. It is doubtful if changes of such a severe nature actually occur in the human enamel organ but it would seem that the metabolic activity of the ameloblast is fairly sensitive to the fluoride ion.

By careful methods of sampling it has been clearly established (BRUDEVOLD, GARDNER and SMITH, 1956; ISAAC *et al.*, 1958) that there is a much higher fluoride

concentration in the enamel surface than in the inner enamel. If there were an inverse relationship between fluoride concentration and degree of calcification it would be expected that the surface layer were least calcified. Yet our micro-radiographs consistently show the surface enamel to be well calcified and the subsurface outer third to be hypocalcified. These findings have subsequently been confirmed by DARLING and BROOKS (1959) who observed diffuse hypocalcification lying beneath a narrow well-calcified surface layer. In severe lesions they have also found the hypocalcified area to involve a greater depth of enamel extending towards the dentino-enamel junction but noted that a well calcified band of enamel always remained at the junction. The following explanation is suggested for this anomolous finding: it is possible that fluoride ions no longer interfere in metabolic processes once they are incorporated into the apatite crystals, but the presence of high concentrations of fluoride in the fluid of the enamel organ may cause a delay in crystal formation. Accordingly, the outer layer may be better calcified than the subsurface enamel because it is in direct contact with tissue fluid for a considerable length of time after the matrix formation has been completed. The uneven calcification related to the striae of Retzius may be associated with rhythmic deposition of inorganic salts and the formation of temporary surfaces which would similarly have a relatively prolonged period of direct contact with the fluid environment.

It is interesting to compare the microradiographic appearance of fluorosed enamel to that of early caries. Longitudinal sections of fluorosed enamel reveal fine radio-lucent lines running at an angle to the striae, and these lines appear similar to those described in relation to natural and artificial lesions (BERGMAN and ENGFELDT, 1954; GUZMAN, BRUDEVOLD and MERMAGEN, 1957; NEWBRUN, BRUDEVOLD and MERMAGEN, 1959). On the other hand, DARLING (1958) and MILLER (1958) consider the decalci-fication of caries to proceed along the prism core, basing their conclusions on findings with transverse sections. If the interprismatic material is the site of relative hypo-calcification of fluorosed enamel, as it appears, then the radiolucency of the prism core seen in caries would be an essential difference between these two lesions. However, this does not explain the similarity seen in microradiographs of longitudinal sections. The final interpretation of these microradiographs may depend upon the radiographic appearance of non-carious, non-fluorosed enamel.

A study of the microdensity tracings of these lesions reveals a difference in the degree of calcification of the surface layer. In the case of an initial carious lesion the outer edge is fairly radiopaque although not as much as the deeper enamel. A sharp drop in radiopacity (increase in photodensity) takes place inside the enamel surface. The fact that the surface layer of enamel in early carious lesions is not as radiopaque as the enamel on the dentinal aspect of the radiolucent area indicates that some demineralization of the surface layer has occurred. Microdensity tracings of roentgenographs of fluorosed enamel indicate that the surface layer is more highly calcified than the remaining enamel (unlike carious enamel) and that there is a radio-lucent zone in the subsurface enamel. The basic difference between these two lesions is that the defects produced by caries occur post-eruptively, whereas the faults in fluorosed enamel are developmental.

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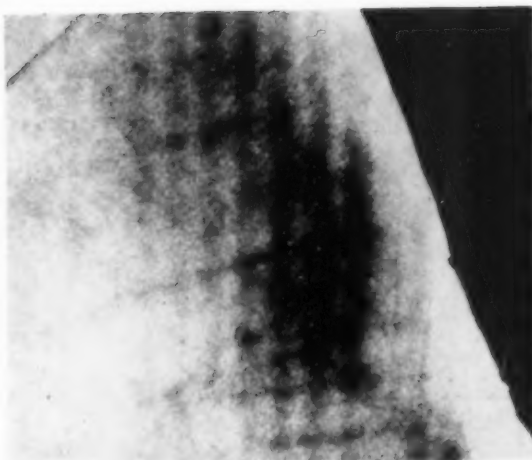
FIG. 1. Microradiograph of fluorosed enamel from Colorado Springs. Note radiolucency of outer third of enamel but well calcified surface layer. ×100.

PLATE 1

STUDIES ON THE PHYSICAL PROPERTIES OF FLUOROSED ENAMEL—II

MICROHARDNESS*

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Abstract—The microhardness was measured on thirty teeth collected from Alamosa, Colorado; Colorado Springs, Colorado; Doland, S. Dakota, and Post, Texas, where the water supply contained 1·5, 2·6, 2·9 and 5·0 p.p.m. of fluoride respectively. The average hardness of teeth from each of these areas was compared with the average hardness of teeth from Birmingham, Alabama (0·00–0·03 p.p.m. F in the drinking water). It was found that the teeth from Colorado Springs and from Post were significantly softer than those from Birmingham but that the teeth from the other locations did not differ significantly. The degree of dental fluorosis appeared to be inversely proportional to the Knoop Hardness.

INTRODUCTION

EARLY investigations of the hardness of enamel and dentine have been reviewed by LEICESTER (1949) and BHUSSRY (1956). Most of the previous studies have been concerned with the hardness of normal enamel, but recently there have been several papers dealing with effect of topical fluoride applications, acids and artificial caries on enamel hardness by PHILLIPS and SWARTZ (1948), CALDWELL *et al.* (1958), NEWBRUN, TIMBERLAKE and PIGMAN (1959). However, no attempt has been made to evaluate the effect on enamel hardness of fluoride taken systemically during the period of tooth development. BOWES and MURRAY (1936) noted that, on grinding fluorosed enamel, it seemed less hard than normal. The purpose of the present investigation was to measure quantitatively the hardness of fluorosed enamel and determine if any correlation exists between the hardness, the degree of dental fluorosis and the quantity of fluoride in the water supply.

MATERIAL AND METHODS

Thirty permanent anterior teeth were collected from Alamosa, Col., Colorado Springs, Col., Doland, S. Dakota and Post, Texas, where the water supply contains 1·5, 2·6, 2·9 and 5·0 p.p.m. of fluoride respectively. Only teeth from persons who had

lived continuously in these locations were included. These teeth were cleaned and mounted in hard inlay wax in individual acrylic resin boxes. The hardness measurements were made using a Kentron microhardness tester with a 500 g load applied for 10 sec. The procedure outlined in a previous paper (NEWBRUN, TIMBERLAKE and PIGMAN, 1959) was observed in determining the Knoop Hardness Number of a tooth.

RESULTS

A total of 344 hardness measurements were carried out on the intact surfaces of thirty teeth used in this experiment. At least ten readings were taken on each tooth, and the average was taken as the Knoop Hardness of the tooth. These values, together with the degree of mottling, the age group, the fluoride concentration of the water supply and the area are shown in Table 1.

TABLE 1. HARDNESS AND MOTTLING OF ENAMEL SURFACE IN RELATION TO FLUORIDE IN THE DRINKING WATER

Tooth No.	Knoop Hardness No. $\pm$ S.D.	Age	Degree of mottling	
F1	333 ± 31		2 m	
F2	291 ± 13	20–29	2 m	
F3	323 ± 37		2 m	
F4	322 ± 42		4 m	Post, Texas
F5	322 ± 17		2 m	F⁻ concentration of water
F6	314 ± 33		2 m	supply, 5 p.p.m.
F7	334 ± 29	30–49	0 m	
F8	335 ± 23		2 m	
F9	269 ± 43		1 m	
Average	305 ± 24		D.F.I.* 189	
F10	341 ± 27		0 m	
F11	324 ± 25		2 m	
F12	322 ± 70	30–49	4 m	
F13	350 ± 15		4 m	Doland, S. Dakota
F14	387 ± 41		0 m	F⁻ concentration of water
F15	372 ± 27		0 m	supply, 2·9 p.p.m.
F16	350 ± 25		1 m	
F17	359 ± 36	20–29	0 m	
F18	367 ± 14		0 m	
F19	373 ± 82		0 m	
Average	355 ± 20		D.F.I. 110	
F20	220 ± 14		2 m	Colorado Springs,
F21	298 ± 18	55	2 m	Colorado
F22	287 ± 21		2 m	F⁻ concentration of water
Average	268 ± 34		D.F.I. 200	supply, 2.6 p.p.m.

TABLE 1—*continued*

Tooth No.	Knoop Hardness No. ±S.D.	Age	Degree of mottling	
F23	400±34		0 m	
F24	383±44	30–49	0 m	
F25	417±54		0 m	Alamosa, Colorado
F26	409±61		0 m	F⁻ concentration of water supply, 1·5 p.p.m.
F27	379±16		0 m	
F28	384±36	20	4 m	
F29	423±39		0 m	
F30	372±32		0 m	
Average	396±17		D.F.I. 50	

* Dental fluorosis index (D.F.I.).

A variable degree of mottling was observed in the teeth used in this experiment. Some teeth appeared normal, others were white and lacking in translucency or showed light or dark brown areas. In assessing the degree of mottling, the classification of VENKATESWARLU, RAO and RAO (1952) was used (Table 2). The Dental Fluorosis Index (D.F.I.) was obtained by totalling m (the grade of mottling), dividing by the number of teeth studied and multiplying by a hundred. The D.F.I. closely resembled the numerical weighted index of clinical severity suggested by Dean (1942) but was on a tooth-unit basis per hundred teeth instead of on a child-unit basis. However, both these fluorosis indices are, at best, semiquantitative measurements. Fig. 1 compares the D.F.I. with the Knoop Hardness of each of the groups studied. It will be noted that there is an inverse linear relationship.

TABLE 2

Grade of mottling	Description of mottling	Valuation
Normal	No mottling	0 m*
Mild	White opacities or patches on the enamel; very faint line (yellow) across the enamel	1 m
Moderate	Distinct brown stain becomes well established	2 m
Severe	Besides the well established brown line, the tooth is worn, edges are chipped off and there is considerable pitting all over the enamel	4 m

* m is the unit of manifestation of fluorine in the form of mottled enamel.

The Knoop Hardness of the nine teeth from Post, Texas, was 305±24, which is significantly lower ($P<0·01$) than values 365±35 and 367±35 obtained (CALDWELL *et al.*, 1957; NEWBRUN, TIMBERLAKE and PIGMAN, 1959) for the Knoop Hardness of

anterior teeth from Birmingham, Ala., where the fluoride content in the drinking water is only 0·00–0·03 p.p.m.

Of the ten teeth from Doland, S. Dakota, the average Knoop Hardness was 355 ± 20, which is not significantly ($P>0\cdot10$) different from the hardness observed for teeth from Birmingham. Unfortunately, the group from Colorado Springs, Col., was very small; only three teeth were available and all were from the same patient (55 years old). They were all moderately mottled and quite soft (Knoop Hardness 268 ± 34). While it is considered that this is the correct hardness value of the teeth examined, it would appear that these teeth are not entirely representative of Colorado Springs. DEAN (1942) studied 404 children in the 12–14 age group from this area and reported an index of fluorosis of 1·3 which would correspond to about 130 D.F.I. The D.F.I. of the teeth whose hardness was measured was 200, which indicates a greater amount of mottling than is usual for this area. In Colorado Springs the water contains 2·6 p.p.m. of fluoride.
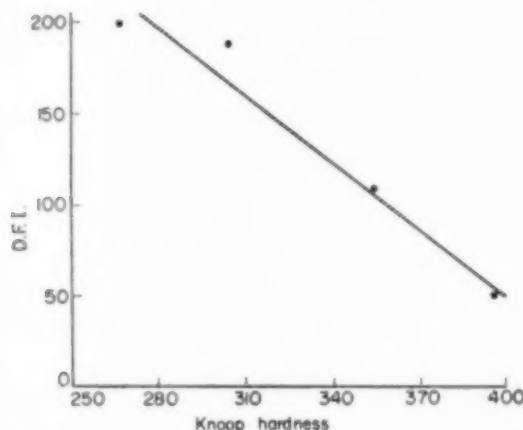
FIG. 1. Relationship of mottling (Dental Fluorosis Index) to Knoop Hardness Number of the enamel.

Eight teeth from Alamosa, Colorado (1·5 p.p.m. of fluoride in the drinking water), averaged 396 ± 18 in hardness. This value is not significantly ($P>0\cdot10$) different from that of Birmingham teeth (367 ± 35). Only one of this group of teeth showed any evidence of mottling.

Almost all fluorosed teeth exhibited pronounced perikymata, much more than had been noted on the surface of non-fluorosed enamel. These irregularities added to the difficulty of obtaining good hardness indentations.

As a further basis of comparison between high and low fluoride areas, the micro-hardness of teeth from Binghamton, New York, (0·05–0·15 p.p.m. F) and Schenectady, New York, (0·05–0·1 p.p.m. F) was measured. The drinking water in these areas, as in Birmingham, is low in fluoride, and the teeth from these localities showed no

evidence of mottling. The results are summarized in Table 3 from which it will be seen that enamel hardness in the low fluoride areas differs only very slightly, being 367 ± 35 for Birmingham, Alabama, 369 ± 30 for Schenectady, New York, and 374 ± 30 for Binghamton, New York.

TABLE 3. AVERAGE HARDNESS OF ENAMEL SURFACE IN RELATION TO DRINKING WATER, COMPARING AREAS WITH LOW AND HIGH FLUORIDE CONCENTRATION

Number of teeth tested	Average Knoop Hardness $\pm$ S.D.	F^- concentration of water supply in p.p.m.	Area
40	367 ± 35	0·00–0·03	Birmingham, Alabama
8	374 ± 30	0·05–0·15	Binghamton, New York
10	369 ± 30	0·05–0·1*	Schenectedy, New York
8	396 ± 18	1·5	Alamosa, Colorado
3†	268 ± 34	2·6	Colorado Springs, Colorado
10	355 ± 20	2·9	Doland, S. Dakota
9	305 ± 24	5·0	Post, Texas

* Schenectady started fluoridation of its water supply in 1952 but due to technical difficulties it has only been carried on intermittently and for a majority of the time it was untreated. As the teeth in this group were all from individuals over 30 years in age the effect, if any, would have been due to topical rather than systemic fluoride.

† All three teeth in this group were obtained from the same individual (55 years old) and showed pronounced mottling.

DISCUSSION

The classical epidemiological findings of DEAN (1942) have shown an extraordinarily precise quantitative relationship between the degree of dental fluorosis and the fluoride concentrations of the public water supply. Subsequent investigations revealed that an inverse relationship also existed between the fluoride content of the water and the dental caries experience of children who consumed the waters throughout tooth development. As an extension of these epidemiological findings, it was shown (ISAAC, BRUDEVOLD, SMITH and GARDNER, 1958a, b) that the amount of fluoride deposited in the enamel during tooth formation and posteruptively was directly proportional to the fluoride content of the water supply and further that enamel solubility was inversely proportional to its fluoride content. The present investigation has shown another correlation, namely the greater the degree of dental fluorosis the lower the Knoop Hardness of the enamel.

These findings appear at first to be inconsistent with established data showing an inverse relationship between fluoride content and enamel solubility. It is a popular misconception that solubility and hardness run parallel. However, the studies of SWARTZ and PHILLIPS (1952) showed no correlation between the initial hardness of

enamel and the amount of phosphorus dissolved by a buffer in a standard time interval. Also workers in these laboratories (NEWBRUN, TIMBERLAKE and PIGMAN, 1959) found no relation between the initial hardness of enamel surfaces and the rate of decrease of hardness. There have been attempts at various times (RICHTER, 1931; HODGE, 1939) to establish a relationship between hardness and caries susceptibility. However, it was learned that the microhardness of sound enamel and dentine does not differ on the average from the microhardness of sound portions of enamel and dentine from carious teeth and that the hardness was independent of the amount of caries in the mouth. It is apparent then that fluorosed teeth can be softer than normal teeth and still remain more resistant to decay.

It has been reported in the literature that a topical application of fluoride solutions to an enamel surface will result in an increased hardness. PHILLIPS and SWARTZ (1948) found an increase of 5·1 per cent with sodium fluoride and 7·1 per cent with stannous fluoride in the hardness of ground enamel following *in vitro* treatment. Similarly HORD and ELLIS (1949) claimed an increase in enamel hardness of 13 per cent following topical applications of sodium fluoride to the teeth of dogs *in vivo*. Recently HERRMANN (1958) and HERRMANN and ROZEIK (1959) have described a two-fold effect (topical and systemic) on the teeth of albino rats following the feeding with very high concentrations of fluoride, 500 p.p.m. or 1000 p.p.m. in the diet. In the rat molars which had already formed they noted an increase in hardness whereas the effect of fluoride on the incisor teeth, which were still forming, was to soften them. The higher the fluoride concentration, the lower was the hardness of the developing teeth. Herrmann suggested that, in systemic fluoride ingestion, calcium fluoride was deposited during mineralization and this was softer than hydroxy-apatite. He explained the increase in hardness of enamel which had already calcified as being due to the formation of fluorapatite. On the other hand, BIBBY and BRUDEVOLD (1954) in reviewing the mechanism of fluoride fixation following topical treatment pointed out that high fluoride concentrations result in deposition of fluoride as calcium fluoride. Lower concentrations of fluoride as occur in the environment of the teeth during formation would lead to deposition of fluorapatite, not calcium fluoride, even in severely mottled enamel. Careful studies in this laboratory have failed to reveal any change in hardness after topical fluoride treatment (NEWBRUN, TIMBERLAKE and PIGMAN, 1959). The results reported in this paper, while not clarifying the situation concerning the effect of topical applications of fluoride on enamel hardness, indicate that high amounts of fluoride taken systemically during the period of tooth development do affect the hardness of enamel.

It is interesting to note that this systemic effect of fluoride on enamel hardness does not become apparent until levels in the water supply exceed 1·5 p.p.m. F⁻. The teeth from Alamosa, Colorado (1·5 p.p.m. F⁻ in the drinking water) were harder, though not significantly, than the teeth from the low fluoride areas and significantly harder than teeth from very high fluoride areas. This suggests that there may be an optimal level of fluoride concentration for enamel hardness as there is for dental caries and mottling, and that this value lies somewhere between 0·15 p.p.m. and 2·6 p.p.m. F⁻.

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HISTOCHEMICAL DISTRIBUTION OF BETA-GLUCURONIDASE IN GINGIVAL TISSUE

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Abstract—The histochemical distribution of β-glucuronidase in gingival tissues of man, rat and mouse was studied using the technique of Fishman and Baker. A positive reaction was found in the epithelium, especially in the basal cell layer. The gingival corium showed a reaction in the blood vessels. The inflammatory infiltrate also showed a positive reaction. The possible histophysiological role of β-glucuronidase is discussed; the topographical location of the enzyme suggests a relationship between β-glucuronidase and cell proliferation.

THE histochemical study of the distribution of enzymes in different tissues has received considerable attention in recent years. Gingival tissues have been explored with reference to a few enzymes. Alkaline phosphatase (ZANDER, 1941; VALLOTTON, 1942; CABRINI and CARRANZA, 1951; TURESKY, GLICKMAN and LITWIN, 1951) has been found to be localized in the vascular components of the gingival corium, increasing in inflammatory and fibrotic processes (CARRANZA and CABRINI, 1952); the epithelium seems to be almost devoid of this enzyme. On the other hand, acid phosphatase has been found in the gingival epithelium (CABRINI and CARRANZA, 1958a); its topographical location suggests a relationship between acid phosphatase and keratinization.

Recently, several techniques for the histochemical demonstration of β-glucuronidase have been developed (SELIGMAN, TSO, RUTENBURG and COHEN, 1954; FISHMAN and BAKER, 1956). The distribution of β-glucuronidase in stratified squamous epithelium has been found to be associated with the basal cell layers (SELIGMAN et al., 1954; FISHMAN and BAKER, 1956; CABRINI and CARRANZA, 1958b) and with the granular layer (MONTAGNA, 1957). A relation between β-glucuronidase and gingival inflammation has been reported (LISANTI and CHAUNCEY, 1957).

MATERIAL AND TECHNIQUE

Ten human gingival biopsies were taken from normal and inflamed areas; oral tissues from stock albino mice and C3H/B.A. mice were also studied. The material was fixed in formaldehyde–chloral hydrate at $4°C$ (FISHMAN and BAKER, 1956). Within 24 hr of the surgical excision, frozen sections were cut and processed according to the Fishman and Baker technique. The substrate, 8-hydroxyquinoline

glucuronic acid, was prepared biosynthetically in the rabbit. All cases were controlled by incubation without substrate, inhibition with potassium hydrogen saccharate and destruction by heat and salts of heavy metals (FISHMAN and BAKER, 1956). The incubation times used were: 20–30 min, 2–3 hr, 6–7 hr and 24 hr.

RESULTS

Results have been very consistent, the only variation being the time of commencement of the reaction, which seems to depend on the fixation time. With short incubation periods (20–30 min) a line of positive reaction was seen in the basal portion of the basal cell layer of the epithelium; the reaction was cytoplasmic, nuclei remaining negative (Fig. 1a). With 2–3 hr of incubation the reaction extended to the lower layers of the epithelium; however, the strongest reaction was still in the basal layer (Fig. 1b). With several hours of incubation (6–7 hr, 24 hr) all the epithelium showed an intensely positive reaction (Fig. 1c).

The corium did not react, except in capillaries where there was an endothelial reaction (Fig. 2), and in inflammatory cells. The cytoplasm of these cells, especially of lymphocytes, gave a strongly positive reaction.

Epithelial areas adjacent to strongly reacting inflammatory infiltrates seemed, in some cases, to have a decreased intensity of reaction (Fig. 3).

DISCUSSION

The results reported agree with previous studies on the distribution of β-glucuronidase in other mucous membranes and skin (FISHMAN and BAKER, 1956; CABRINI and CARRANZA, 1958b). The strongest reaction is localized in the basal cell layer, where the proliferative activity of the cells is most marked.

CHAUNCEY, LIONETTI, WINER and LISANTI (1954) found that human saliva contains β-glucuronidase. SCHULTZ-HAUDT and SCHERP (1954) have shown that the viridans streptococci isolated from gingival pockets are capable of producing β-glucuronidase. LISANTI (1958) has suggested that β-glucuronidase in gingival tissue may be partly of mammalian origin and partly of bacterial origin; he found that normal gingiva does not contain enzyme of bacterial sources, but that inflamed tissue does.

In the present investigation it was found that the corium reacts weakly, except in inflamed areas where the reaction was stronger. The reduction of enzymatic activity in epithelial areas adjacent to strongly reacting inflammatory infiltrates may be related to (1) a reduction in the available substrate due to the proximity of a strongly reacting area which would reduce the concentration of the substrate in the epithelial areas, or (2) a true decrease in enzymatic content of the epithelial tissue.

β-Glucuronidase has been related to detoxification of phenolic compounds. FISHMAN (1947) has reported a variation in the content of this enzyme in the endometrium and related it to the production of sex hormones. A greater amount of this enzyme was found in tissues undergoing a neoplastic proliferation than in normal tissues (FISHMAN, ANLYN and GORDON, 1947). This might point to a relation between β-glucuronidase and cell proliferation, which would explain the presence of the

enzyme in the basal cell layer of gingival epithelium and other stratified squamous epithelia.

It seems of interest to compare the distribution in gingival tissue of acid phosphatase and β-glucuronidase. They are both located mainly in the epithelium, the former in the superficial layers and related presumably to keratinization, the latter in the basal layers and probably related to cell proliferation.

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FIG. 1. β-Glucuronidase in human gingiva, Fishman and Baker technique. ×108. Note the increasing intensity of the reaction with different incubation times. (a) 30 min; (b) 2 hr; (c) 24 hr.

FIG. 2. β-Glucuronidase in human gingiva, Fisherman and Baker technique. ×122. Note the positive reaction in blood vessels and in basal cell layer of epithelium. Incubation time 2 hr.

FIG. 3. β-Glucuronidase in human gingiva, Fishman and Baker technique. ×122. Same case as Fig. 2. Note the lack of reaction in the epithelial tissue adjacent to inflamed corium. Incubation time 2 hr.
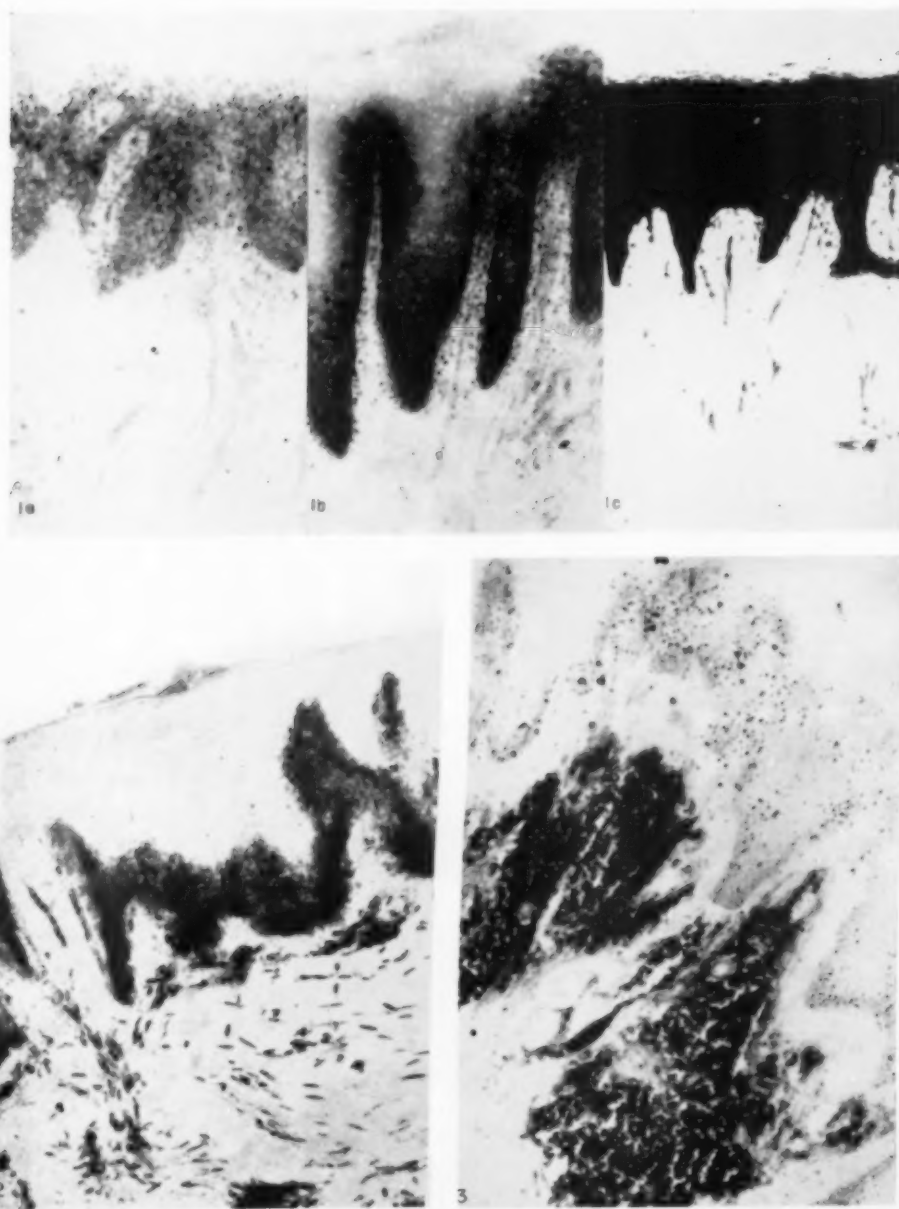
PLATE 1

THE SPECTROCHEMICAL ANALYSIS OF METALS IN RAT MOLAR ENAMEL, FEMURS AND INCISORS

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Abstract—A quantitative a.c. arc salt cap method using 20 mg of molar enamel has been devised for the determination of cobalt, copper, iron, manganese, nickel and zinc in the range of 1–300 p.p.m. Rat molar enamel in powder form is dissolved in HCl; the resulting solution is dried on graphite electrodes and introduced into an a.c. arc discharge. Femurs and incisors are oxidized with nitric acid and treated similarly. Use of an Ilford Q2 ultra-violet sensitive plate has demonstrated greatly improved sensitivity for zinc in this matrix to 0·5 p.p.m. using the 2138·56 Å line. Average precision is within 10 per cent of the amount present. Analytical results are indicated and correlation of metal uptake in enamel with metal-rich diet demonstrated.

SPECTROGRAPHIC techniques have been used in qualitative analysis of dental tissue by DREA (1936) and by LOWATER and MURRAY (1937). Quantitative determinations of copper, lead and tin were made by BRUDEVOLD and STEADMAN (1955, 1956a,b) and for a variety of trace elements in ancient teeth by STEADMAN *et al.* (1959) using d.c. arc excitation.

In connection with a project attempting to correlate metal content in enamel with metal-rich diet and dental caries, the present study sought to determine cobalt, copper, manganese, nickel, zinc and iron in rat molar enamel in the range of 1–300 p.p.m. Analysis of femurs and incisors from the same animals was also accomplished.

MATERIALS AND METHODS

A. *Equipment and standards*

Excitation was obtained by use of a 2200 V, 4·4 A a.c. arc discharge (OWENS, 1939). A Baird-Atomic three meter grating spectrograph with a reciprocal dispersion of 5·5 Å/mm in the first order was used. Eastman Spectrum Analysis No. 2 plate was used to record 2400–3900 Å. An ultra-violet sensitive Ilford Q2 plate (available from Spex Industries, Inc., 205 Jamaica Avenue, Hollis 23, N.Y., U.S.A.) was used to record wavelengths shorter than 2400 Å when improved zinc sensitivity was needed, utilizing the 2138·56 Å line.

Standards were prepared containing 2% $Ca_3(PO_4)_2$. Highest purity material was obtained by reacting appropriate amounts of Mallinckrodt's reagent grade $CaCl_2$ with C.P. grade H_3PO_4. Molybdenum and bismuth were added as internal standards. Desired standard concentrations from 1 to 300 p.p.m. were prepared by taking 8 ml

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aliquots of a 80 ml volume containing 2·0 g of $Ca_3(PO_4)_2$ and adding metals of known concentrations to give final concentrations in 10 ml based on the $Ca_3(PO_4)_2$ present.

B. *Procedure*

Enamel samples were obtained by grinding one-third to one-half the thickness of the enamel from the buccal and lingual surfaces of the molar teeth. The evolved dust was collected in a small electrostatic precipitator (HENDERSHOT, 1959). Pooled samples of 15–60 mg were obtained in this manner from groups of four to fifteen Wistar rats.

Metal levels of pooled samples of the incisor teeth were determined on the entire erupted portions. No attempt was made to separate the incisor enamel from the dentine. Pooled samples of femur shafts were obtained by removing sections 3–4 mm in length from the central portions of the shafts.

In the metal feeding experiments, the animals were placed on the experimental and control diets at 21 days of age and sacrificed on the 161st day of life. The diet consisted of Purina Laboratory chow and sucrose in a 1:1 ratio. The teeth were sectioned antero-posteriorly in a vertical plane with a diamond disk and examined for caries at ×20 magnification. Samples of enamel for analysis were obtained before the teeth were examined for carious lesions.

The enamel sample was dissolved in a 20 per cent by volume HCl solution containing molybdenum and bismuth as internal standards, to give a final concentration of 1 mg/0·05 ml (20 mg/ml). Triplicate runs were made on the sample thus prepared by placing 60 μl on the ends of kerosene-impregnated pure graphite electrodes $\frac{3}{8}$ in. long and $\frac{1}{4}$ in. in diameter. The solution was dried on the electrode tips over gas microburners. The electrodes were then introduced into an a.c. arc discharge using the following exposure conditions:

Spectral region, Å	1900–3900
Slit width, μ	25
Preburn period, sec	5
Exposure time, sec	45
Analytical gap, mm	2

Samples of femur shafts and the erupted portions of the lower incisors received similar spectrographic treatment. After wet washing the organic matter with nitric acid, the femur and incisor samples were concentrated by factors of 1·5 and 2·0, respectively, to provide approximately comparable amounts of salt on the electrodes. The residues were taken up in a 20 per cent by volume HNO_3 solution containing molybdenum and bismuth and compared with standards prepared with HNO_3 instead of HCl.

Standard developing and calculating procedures were followed with transmittance readings being taken on a non-recording microphotometer.

Analytical curves were prepared from data obtained from the described standards. Standards were included on each plate to check the curves. Table 1 lists the analytical line pairs used with the concentration ranges over which they are useful. Spectral background was subtracted where indicated.

TABLE 1. ANALYTICAL LINE PAIRS

Element	Analytical line (Å)	Internal standard line (Å)	Concentration range (%)
Co	3453·50	Mo 3193·97	0·00025–0·02
Cu	3273·96†	Mo 3193·97	0·0001 –0·01
Mn	2801·06†	Mo 3193·97	0·0001 –0·03
Mn	2933·06	Mo 3193·97	0·0001 –0·03
Ni	3050·82	Mo 3193·97	0·0001 –0·03
Ni	3414·77	Mo 3193·97	0 0001 –0·03
Zn	3282·33†	Mo 3193·97	0·0010 –0·03
Zn	3345·02*	Mo 3193·97	0·0010 –0·03
Zn‡	2138·56	Bi 2228·25	0·00005–0·003
Zn‡	2061·91	Bi 2061·70	0·0005 –0 03
Zn‡	2025·15	Bi 2061·70	0·0001 –0·03
Fe	3021·07†	Mo 3193·97	0·0005 –0·03

* Background subtracted.
† Background subtracted only with HNO₃ solutions.
‡ Ilford Q2 plate.

RESULTS

Use of the Ilford Q2 plate and the Zn 2138·56 Å line provided greatly improved sensitivity enabling detection of as little as 0·5 p.p.m. of zinc in the calcium phosphate matrix. Using S.A. No. 2 plate and Zn 3345·02 Å or Zn 3282·33 Å sensitivity for zinc was 10 p.p.m.

Other zinc lines at 2025·51 Å and 2061·91 Å may also be used for higher concentrations at which the 2138·56 Å line reverses. Samples run as the nitrate instead of the chloride produced measurably weaker spectra. Use of a 25 cm instead of a 35 cm focal length lens compensated for this effect.

TABLE 2. ANALYTICAL PRECISION DATA

Element	Concentration (p.p.m.)	Standard deviation	C.V.* (%)
Mn	10	0·72	7·2
	25	2·18	8·7
	35	1·72	4·9
Zn	25	1·84	7·3
	35	1·94	5·5
Cu	10	2·0	20
	25	3·2	13
Ni	10	1·58	16
	25	5·1	20

* C.V. = coefficient of variation = $\dfrac{\text{standard deviation}}{\text{average concentration in p.p.m.}} \times 100$.

Table 3.   Control metal levels in molar enamel, incisor and femur shaft of the rat

Tissue	No. of animals	Sex	p.p.m.					
			Ni	Zn	Cu	Co	Mn	Fe
Enamel	10	M	3·6	13	6	<2·5	36	112
	15	M	4	9	1·4	<2·5	55	90
	7	M	15	30	26	<2·5	38	~120
	6	M	6	15	13	<2·5	58	~200
	5	M	4·8	23	12·5	<2·5	62	~110
	8	M	2·7	8	2·4	<2·5	110	63
	11	F	2·6	14	7	<2·5	37	33
	12	F	5	11	3	<2·5	60	132
$n_t = 83$	9	F	2·5	13	2·2	<2·5	70	35
Mean			4·76	13·8	6·69	<2·5	57·2	94·3
S.E.†			1·77	4·6	2·76		19·9	33·9
Incisor	10	M	<0·5	17	0·7	<1·3	3·5	71
	8*	M	<0·5	29	<0·5	<1·3	0·9	175
	8*	M	<0·5	27	<0·5	<1·3	1·9	200
	11	F	<0·5	18	<0·5	<1·3	2·3	78
	12	F	<0·5	28	<0·5	<1·3	1·0	195
$n = 57$	8	F	<0·5	27	<0·5	<1·3	1·0	165
Mean			<0·5	24·0	<0·5	<1·3	1·79	144
S.E.†				9·6			0·67	18
Femur	10	M	<0·7	57	0·7	<1·7	<0·7	5
	15	M	<0·7	52		<1·7	<0·7	5
	8*	M	<0·7	64	0·8	<1·7	<0·7	10
	8*	M	<0·7	58	0·5	<1·7	<0·7	14
	8	M	<0·7	57	0·9	<1·7	<0·7	8
	11	F	<0·7	45	0·8	<1·7	<0·7	5
	12	F	<0·7	58	2·0	<1·7	<0·7	5
	2*	F	<0·7	64	1·1	<1·7	<0·7	13
	8*	F	<0·7	92	0·8	<1·7	<0·7	14
$n = 91$	9	F	<0·7	59	0·7	<1·7	<0·7	5
Mean			<0·7	58·9	0·78	<1·7	<0·7	7·46
S.E.†				18·9	0·32			2·42

* Samples were obtained from rats of two different colonies of the Wistar strain. Asterisks identify samples from rats of one of the colonies.

† S.E. = standard error = $\dfrac{\text{standard deviation}}{\sqrt{n}}$. n_t = total no. of animals.

Certain precautions must be taken when using the Ilford Q2 plate. The emulsion on this plate is susceptible to scratching or marring and must be handled carefully.

Plates from different boxes were found to vary considerably in emulsion sensitivity. Photographic processing of the plate was identical with that for S.A. No. 2 plates.

Another precaution which merits attention pertains to the time of drying of the electrodes before arc analysis. Uncontrolled or varying times of drying in an oven or desiccator box have been found to yield considerable shifting in analytical curves. This alteration is apparently caused by a change in the state of hydration or composition of the compounds present. Standards left in an oven at 100°C for 2 hr before running gave reproducible analytical curves while standards left overnight in the oven at about 100°C showed considerable analytical curve displacement when compared with the curves prepared after 2 hr drying. Thus in order to obtain constant analytical curves, uniformity in procedure must be followed.

TABLE 4. METAL FEEDING, DENTAL CARIES AND METAL UPTAKE
BY ENAMEL IN THE MALE RAT

Agent	p.p.m. metal added to diet	Carious lesions animal[†]	p.p.m. metal in enamel			
			Zn	Ni	Mn	Cu
Control	0*	4·1[13]	23	10	48	19
ZnSO$_4$	85	6·3[6]	46	7	68	7
	170	4·2[4]	61	5	56	14
	340	8·3[6]	80	7	77	8
	680	8·6[7]	111	7	58	9
NiCl$_2$	93	6·4[7]	17	21	89	—
	185	7·8[5]	21	24	106	11
	370	4·7[6]	15	32	86	8
	740	3·9[7]	21	63	—	10
Mn-acetate	85	2·8[4]	20	2	140[‡]	6
	170	5·4[7]	20	3	200	3
	340	0·4[7]	21	22	200	7
	680	2·3[4]	18	13	>200	6
CuSO$_4$	95	4·2[5]	26	6	67	11
	190	3·4[5]	22	7	40	16
	380	10·5[4]	17	5	88	12
	760	6·4[5]	16	1	59	9

* Control diet (Purina Laboratory Cnow, 50 per cent; sucrose, 50 per cent) contained 3, 0·5 and 22 p.p.m. of Zn, Ni and Mn, respectively.

† Superscript—number of animals in group. By analysis of variance, there is no significant inhibition of dental caries among the five groups. ZnSO$_4$ caused 76 per cent increase in caries incidence which is significant at the 5 per cent level.

‡ Mn values approximated from an extrapolated curve.

Table 2 illustrates analytical precision obtained on replicate runs of selected standards in HNO$_3$. Standards at 10 and 25 p.p.m. levels were run fifteen times and the 35 p.p.m. standard run nine times to give tabulated results.

Five replicate runs, each of HCl standards at the 5 and 10 p.p.m. level gave coefficients of variation for nickel of 6·2 and 5·5 per cent respectively, suggesting better reproducibility possible than indicated in Table 2 for HNO_3 standards for this element.

Table 3 summarizes results obtained in molar enamel, femurs, and incisors of control animals. As indicated, nickel was detected in enamel but not in incisors or femurs; manganese was not detected in femurs. Cobalt was not detected in any sample. The following mean values (p.p.m.) were obtained from enamel, incisor and femur shaft, respectively: Ni, 4·76, <0·5, <0·7; Zn, 13·8, 24·0, 58·9; Cu, 6·69, <0·5, 0·78; Mn, 57·2, 1·79, <0·7; Fe, 94·3, 144, 7·46.

Table 4 illustrates the graded uptake of zinc, nickel and manganese in molar enamel with increasing concentration in the diet when manganese was fed as the acetate, nickel as the chloride and zinc as the sulphate. Manganese values above 100 p.p.m. exceeded the levels of included standards and were estimated from an extrapolated curve. Enamel levels of copper were not increased by feeding copper sulphate. The significance of the data pertaining to carious lesions is indicated in Table 4.

DISCUSSION

The solution technique and the a.c. arc provide greater uniformity in sampling than obtained by using milligram quantities of powdered enamel and a d.c. arc. The method described also provides improved sensitivity for zinc and copper than that reported by STEADMAN et al. (1959) using the same spectral lines. Zinc sensitivity has been improved from 60 to 10 p.p.m. on S.A. No. 2 plate and copper from 5 to 1 p.p.m.

Careful control of, and uniformity in time and temperature of, sample drying is necessary to prevent hydration problems.

This work has demonstrated that the Ilford plate provides greatly improved sensitivity for zinc in this matrix utilizing Zn 2138·56 Å. Other work in this laboratory has also found it valuable in determining zinc in organic samples down to 0·2 p.p.m. using a similar salt cap method with a sodium nitrate matrix.

Although the method was specifically designed for the determination of six elements, it could well be extended to include any other metals or metalloids normally determined by spectrographic means.

In addition to providing data on normal trace metal levels in rat tooth and bone, these studies have demonstrated a graded uptake of metals by the dental enamel of the rat. Assimilation of zinc, nickel and manganese clearly exhibits a dose–response pattern. However, there is some specificity of metal uptake by the enamel of the rat, for copper levels, unlike those of zinc, nickel and manganese, are not increased by metal feeding. While it is beyond the scope of this paper to draw definite conclusions on metal interrelationships pertaining to uptake and the effect of metal uptake on the incidence of dental caries, the data do provide a basis for future studies. A preliminary report of an extension of this work has been made (HENDERSHOT, FORSAITH and MANSELL, 1959).

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HISTOCHEMICAL OBSERVATIONS ON THE RIBONUCLEIC ACID AND GLYCOPROTEIN CONTENT OF THE OSTEOCLASTS OF THE NORMAL AND *ia* RAT

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Abstract—The histochemically demonstrable ribonucleic acid (RNA) and glycoprotein content of the osteoclast has been investigated in both normal and *ia* rats. The osteoclasts of the normal animal vary considerably in the amount of RNA present in their cytoplasm. Thus, the cells can be arranged in a series based on a progressive decrease or increase in RNA content. The majority of the osteoclasts contain an oxyphilic and pyroninophobic zone of variable width adjacent to the bone. It is suggested that the variation in RNA content might possibly be correlated with the functional state of the cell.

The osteoclasts of the *ia* rat differ from those of the normal rat in the pattern of their RNA distribution. The predominating number of cells lack the oxyphilic, pyroninophobic zone adjacent to the bone. Since comparable results were obtained with methylene blue, pyronin and gallocyanin, it is felt that the overall decrease in thionin-positive material may be due to dye–protein interactions. The glycoprotein of the normal osteoclast is confined to sparsely scattered granules. A marked increase in the number of glycoprotein granules occurs in many of the *ia* osteoclasts. The significance of this increase is not apparent.

INTRODUCTION

THE osteoclast is generally thought to arise in response to a humoral or mechanical stimulus and spend its short life-span modelling the bone. It is surprising, therefore, to find a large population of osteoclasts in the bones of the *ia* strain of rats—a strain which is characterized by severe impairment in bone resorption (SCHOUR, BHASKAR, GREEP and WEINMANN, 1949). This negligible bone resorption in the presence of many osteoclasts may be the result of a number of factors. Important among these may be the inability of the osteoclasts to perform their normal function because of a deficiency in some cellular component.

Observations on the histochemistry of the osteoclast have not been as extensive as desirable when one considers the importance of the osteoclast in the process of bone resorption. HELLER-STEINBURG (1951) has described granules in the cytoplasm of the osteoclast which stain red after periodic acid–Schiff (PAS) treatment and are thought to be granules of glycoprotein. These granules were present during rickets and healing rickets and were not decreased after treatment with parathyroid extract. KROON (1954) also reported that the striated border and some of the row of vacuoles

gave a positive reaction with PAS. The increase in the number of vacuoles and their final loss of staining was believed to be related to the functional state of the osteoclast. BHASKAR, MOHAMMED and WEINMANN (1956) found that the thionin-positive, ribonuclease-digestible material occurring in the cytoplasm of the normal osteoclast is reduced in amount in the osteoclast of the *ia* rat. These results suggested the possibility that protein synthesis is decreased in the *ia* osteoclast.

The present study was undertaken to determine by histochemical methods in what way the osteoclasts of the *ia* rat differ from those of the normal rat in their intracellular content of ribonucleic acid and glycoprotein.

MATERIALS AND METHODS

Animals from our own colony of *ia* and normal rats were sacrificed by cervical subluxation at 4 days of age. The right mandible, the left mandible, the right maxillary area and left maxillary regions were removed and placed in appropriate fixatives. Paraffin sections cut at 5 μ in a parasaggital plane to include the upper or lower incisor, the three molars and the surrounding tissues were used in the histochemical analysis.

Ribonucleic acid. Typical tinctorial reactions of RNA include (1) extinction of staining with methylene blue below the level of pH 4, (2) positive staining with pyronin, gallocyanin and thionin and (3) removal of the positive basophilic staining materials by ribonuclease. These several methods were used to determine the RNA content of the osteoclast.

Sections of tissue fixed in Zenker–acetic or Helly's fluid were stained with eosin–methylene blue (EMB) at a pH of 5, or with 5×10^{-4}M methylene blue in dilute buffers at various pH levels from 2 to 7 (DEMPSEY, BUNTING, SINGER and WISLOCKI, 1947). Other sections of tissue fixed in Carnoy's fluid, 10% formaldehyde or formol–acetic–alcohol were stained with methyl green–pyronin (TAFT, 1951), gallocyanin–chrome-alum (PEARSE, 1953) or thionin (LAVELLE, 1951; BHASKAR, MOHAMMED and WEINMANN, 1956). In each instance additional slides were incubated with ribonuclease (Worthington) at a concentration of 1 mg/ml in water or saline for 1–2 hr at 37 or 65°C.

Glycogen and glycoprotein. Sections of material fixed in Bouin's fluid were treated with one of the following: Schiff reagent, periodic acid–Schiff (PAS), periodic acid–Schiff after salivary digestion, or Gomori's aldehyde fuchsin. The latter included slides stained without oxidation as well as other slides that had been oxidized previously with either acidified permanganate or peracetic acid (LILLIE, 1954).

RESULTS

Normal rat

Bone adjacent to the embryonic tooth contains a large number of osteoclasts active in modelling the bone to provide for the expansion of the developing tooth germ. The osteoclasts are located along the edges of well-defined bony trabeculae. They vary in size from uninucleated cells of a few microns in diameter to multi-nucleated cells measuring about 150 μ (Fig. 1). The nuclei tend to be crowded to

the side of the cell away from the bone and contain one or two nucleoli. The cytoplasm is irregular in outline, coarsely granular and contains varying numbers of vacuoles. A striated border can be seen in some cells immediately adjacent to the bone. This border is usually confined to an area somewhat less than the length of the contact between cell and bone.

When a series of sections of normal tissue is stained with methylene blue at different pH levels, cytoplasmic basophilia in the osteoclast appears at pH 2 and increases markedly in intensity at pH 5 (Fig. 2). Ribonuclease removes the positive staining material. The striated border and the vacuoles are unstained throughout the pH range from 2 to 7. In the majority of osteoclasts, basophilia is confined to granules and short thread-like structures. These are numerous in the cytoplasm adjacent to the deeply blue nuclei and sparse elsewhere. Considerable variation exists between the individual osteoclasts in the amount of this basopilic material. At any pH level, some of the osteoclasts will be completely devoid of and others replete with basophilic material.

The intracellular distribution of the granular material is more sharply defined in the osteoclasts stained with EMB at pH 5 (Fig. 3). The cytoplasm shows two fairly distinct areas characterized by the staining reactions of the cytoplasmic granules: an area nearest the striated border contains a preponderance of eosinophilic granules, and another surrounding the nuclei contains a preponderance of basophilic granules. Moreover, in the total population of osteoclasts, there are cells with the cytoplasm completely filled with either basophilic or eosinophilic granules or containing varying proportions of the two varieties. In the latter case, the oxyphilic zone is always located in the cytoplasm adjacent to the bone. The striated border stains a vivid red but the "vacuoles" remain unstained. These observations indicate a considerable range in cytoplasmic reaction from complete basopilia to complete oxyphilia. A study of serial sections reveals that the stainability reflects a true variation within the cytoplasm of the osteoclasts and not a false picture due to the level at which the individual cell might have been cut at the time of sectioning.

Granular material which stains with pyronin (Fig. 5), gallocyanin or thionin is also concentrated on the side of the cell away from the bone. With each staining procedure, variations occur in the amount of positive-staining material in individual cells. The "phobic" zone, when present, is adjacent to the bone. Ribonuclease removes the positive-staining material.

The basophilic granulation of the osteoclast being of primary interest, the cells were subjectively divided into five categories based roughly on the percentage of the cytoplasm filled with basophilic granules. The results of a count of 100 cells in an EMB preparation are recorded in Table 1. Comparable results were obtained with the other basophilic reactions. It is quite evident from these figures that the osteoclasts tend to be distributed in a normal curve with the greatest number of cells at the 50 per cent level.

The outstanding finding in the sections treated with PAS is the presence of spherical, intracytoplasmic granules which stain a reddish colour (Fig. 7). These granules fail to stain without previous oxidation with periodic or peracetic acid.

They continue to stain following salivary digestion. These procedures eliminate the uncomplexed aldehydes and glycogen as possible cytoplasmic components; there is reason to believe that the granules contain the polysaccharide–protein complex, glycoprotein (HELLER-STAINBERG, 1951). The osteoclasts often contain sparsely scattered granules but occasional cells are filled with uniform-sized, spherical bodies. The "vacuoles" and the striated border failed to stain and no glycogen *per se* was found in the osteoclasts. .

TABLE 1.   THE NUMBER OF OSTEOCLASTS OF EACH CELL
TYPE FOUND IN A COUNT OF 100 CELLS

% Granulation	100	75	50	25	0
Normal rat	8	16	42	27	7
ia rat	15	58	24	2	1

Gomori's aldehyde–fuchsin following oxidation with acidified permanganate or peracetic acid reveals granules of similar size and distribution (Fig. 9). Without previous oxidation, the osteoclasts fail to show any positive-staining material.

Results in the ia *rat*

The osteoclasts of the *ia* rat are found along edges of bone and also lying free in the connective tissue. The bone itself is thicker than normal due to the reduced rate of resorption. At many sites this results in direct contact between the expanding tooth germ and the bone. Osteoclasts usually cluster about these areas of contact. Although there seems to be a predominance of the larger-sized cells, the osteoclasts are generally similar in size, distribution and morphological characteristics to those found in normal animals.

The basophilic granules exhibit the same histochemical reactions as the normal, but there is a marked difference in the overall pattern of distribution. Most of the osteoclasts are filled with basophilic granules and lack the zone adjacent to the bone which in the normal animal contains a sparse basophilic and heavy oxyphilic granulation. In sections stained with EMB at pH 5, very few of the cells are entirely eosinophilic (Fig. 4). The pyronin- and gallocyanin-positive materials follow a similar pattern (Fig. 6). The various types of osteoclasts found in the *ia* rat are identical in quantity and quality of granulation to osteoclasts seen in the normal rat. It is only in the proportions of the cell types present that a difference appears. A count of 100 osteoclasts per rat (Table 1) shows that approximately three-quarters of the cells in *ia* rats have more than half of their cytoplasm filled with basophilic granules, whereas for normal rats the figure is more nearly one-quarter.

The thionin-positive material is reduced in amount in the *ia* osteoclast. The number of granules staining dark blue (orthochromatic) or violet (metachromatic) after exposure to the thionin solution is decreased. However, the reaction among the osteoclasts is not uniform.

The glycoprotein granules of the osteoclasts of the *ia* rat differ from those of the normal rat in one respect. They are more numerous in both the PAS and aldehyde fuchsin preparations (Figs. 8, 10).

DISCUSSION

The histochemical criteria for the identification of RNA comprise: (1) extinction of staining with methylene blue below pH 4; (2) positive staining with pyronin, gallocyanin and thionin; and (3) the removal of the positive-staining material in each instance by ribonuclease (PEARSE, 1953). Using these criteria, we have found that the osteoclasts of the normal rat vary considerably in the amount of RNA present in their cytoplasm. The cells can be arranged in a series based on their RNA content. At one extreme are cells with heavy concentration of RNA throughout their cytoplasm and at the other extreme are cells completely devoid of RNA. Between these extremes lie the majority of osteoclasts with RNA concentrated in a zone of cytoplasm away from the area of contact with bone.

Whether the variation in RNA content can be correlated with the functional state of the cell is an intriguing possibility. However, any serial arrangement of progressive changes is necessarily subjective when knowledge is confined to the static picture presented in normal tissue at the time of sacrifice. It is tempting, nonetheless, to draw a parallel between the diminution of RNA content in this arbitrary series of osteoclasts and the rapid decrease in RNA of nerve cells under intense stimulation (CASPERSSON, 1947). Some elucidation of the problem may be obtained from investigations, currently under way, in which osteoclastic activity is altered by various experimental conditions.

That some relationship may exist between cell function and RNA content is supported by findings on RNA content of osteoclasts of the *ia* rat. These findings, with exception of the thionin reaction, indicate that only the pattern of distribution differs from that of the normal. Most osteoclasts have RNA positive granules throughout their cytoplasm rather than confined to a zone adjacent to the bone. The significance of these observations is obscure and confused by the thionin-stained material. The reduction of thionin-positive, ribonuclease-digestible material is considered by BHASKAR, MOHAMMED and WEINMANN (1956) as a possible indication that protein synthesis is deficient in the *ia* osteoclast. It may be that the thionin reaction is due to the protein–dye interrelationships of a metachromatic dye (BERGERON and SINGER, 1958). Whether this or some other explanation is more correct, the evidence supplied by the use of additional specific reactions of RNA, especially pyronin (PEARSE, 1953), shows that the *ia* osteoclast is not deficient in RNA. In fact, the cells clearly contain an adequate supply of materials for the synthesis of protein. Speculation on the questions raised by these studies can perhaps best await further investigation of the fluctuations in RNA content in relation to the metabolic activity of the osteoclast.

The presence of glycoprotein in cytoplasmic granules of the osteoclast was established by the staining reactions with PAS and aldehyde fuchsin. SCOTT and CLAYTON (1953) have shown that both Schiff reagent and aldehyde fuchsin have an

affinity for aldehyde groups. However, aldehyde fuchsin differs from Schiff reagent in that it also possesses an affinity for strong sulphuric acid esters. The latter stain with the dye before any oxidative procedures are carried out. Since the osteoclasts in sections of both normal and *ia* rats failed to show any positive staining without previous oxidation, the group of substances classified as strong sulphuric acid esters can be eliminated as possible cytoplasmic components. The aldehydic group responsible for positive staining is probably derived from a polysaccharide combined with a protein. At the present time it is suspected that the aldehyde fuchsin and the Schiff reagent are reacting with the same cytoplasmic component, glycoprotein.

There is a possibility that the glycoprotein granules are indicative of secretory activity. During the elaboration of bone, glycoprotein is present in the osteoblast and osteocyte (HELLER-STEINBERG, 1951) and a similar correlation exists between enamel formation and the ameloblast (ENGEL, 1948; BEVELANDER and JOHNSON, 1955). If these granules are secretory antecedents, it is difficult to explain their presence in the osteoclast. Glycoprotein occurs in many but not all of the osteoclasts of the normal rat and in increased amounts in the osteoclasts of the *ia* rat. These observations would suggest at least two possibilities, either the osteoclast is a secretory cell or glycoprotein is involved in metabolic activities other than that of secretion. It seems reasonable to assume that the demonstrable glycoprotein may decline as the activity of the cell increases.

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FIG. 1. Normal rat. Osteoclasts along bone at side of embryonic tooth. Note
variations in staining. EMB. ×430.

FIG. 2. Normal rat. Osteoclasts with decreased granulation adjacent to row of
vacuoles. MB pH5. ×980.

FIG. 3. Normal rat. Osteoclasts showing granulation surrounding nuclei which
is blue (RNA) and adjacent to bone which is red in slide preparation. Striated border
evident in cell at top. EMB. ×980.

FIG. 4. *ia* Rat. Osteoclasts with predominantly blue granules (RNA). Cells at
lower right comparable to normal, see Fig. 3. EMB. ×980.
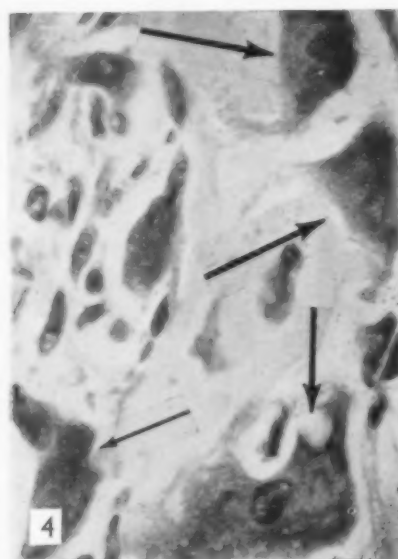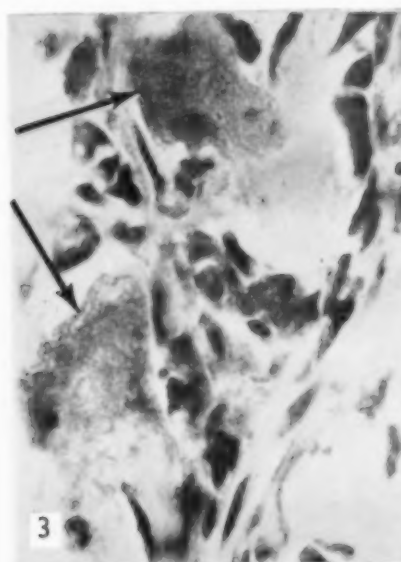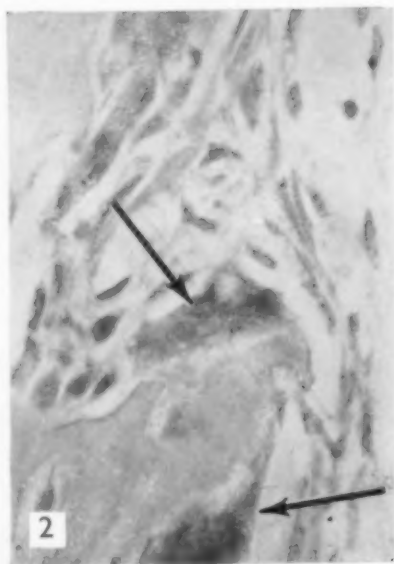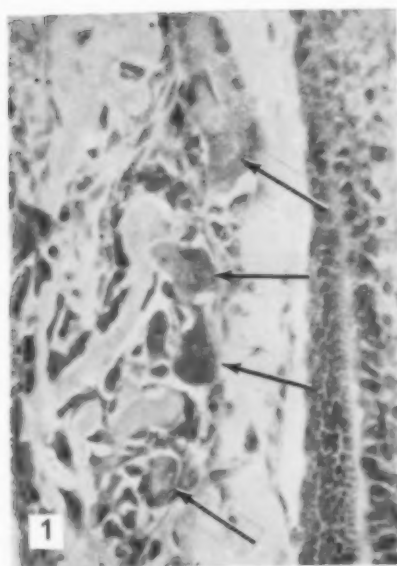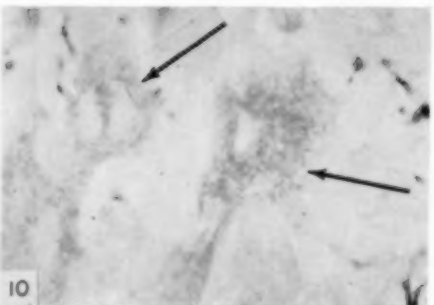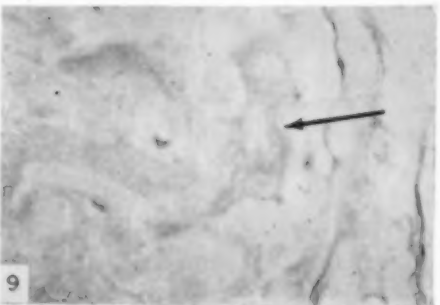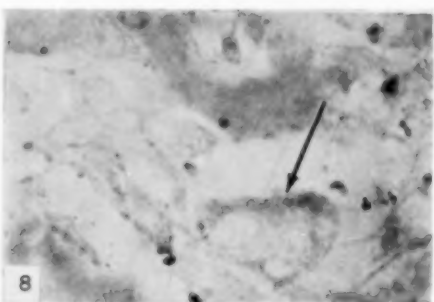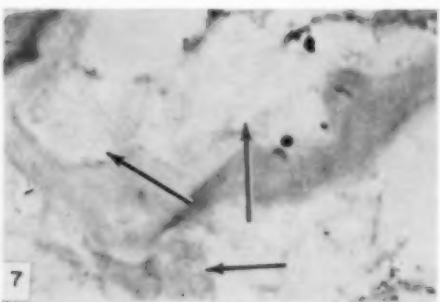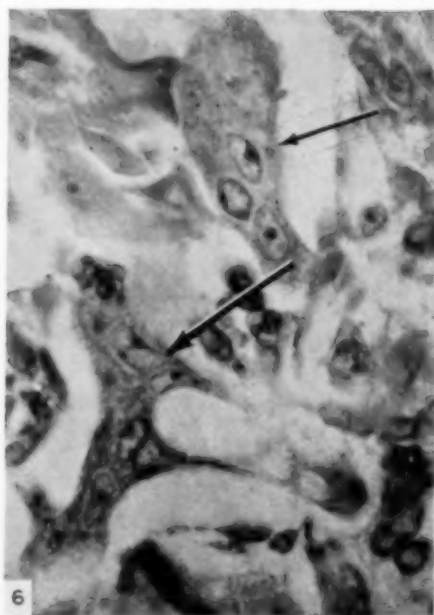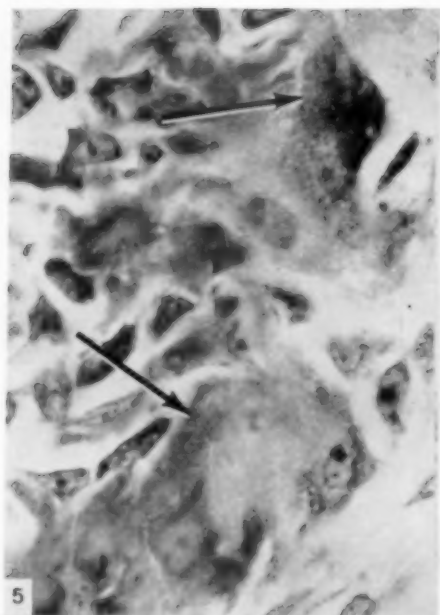
PLATE 1

PLATE 2

FIG. 5. Normal rat. Pyronin-stained granules concentrated in nuclear region of osteoclast. Methyl green–pyronin.   ×980.

FIG. 6. *ia* Rat. Osteoclasts with pyronin-positive granules throughout cytoplasm. Methyl green–pyronin.   ×980.

FIG. 7. Normal rat. Osteoclasts showing granules of glycoprotein. PAS.   ×980.

FIG. 8. *ia* Rat. Osteoclast exhibiting increase in glycoprotein granulation. PAS. ×980.

FIG. 9. Normal rat. Osteoclast with faint, scattered granules. Aldehyde–fuchsin. ×980.

FIG. 10. *ia* Rat. Greater number of granules in osteoclasts, compare with Fig. 9. Aldehyde–fuchsin.   ×980.

pH ON THE TEETH OF ALBINO RATS UNDER VARIOUS CONDITIONS CONDUCIVE TO DENTAL CARIES

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Abstract—The pH on the teeth of albino rats with reference to the time of eating was obtained by training the animals to eat their food allowance at scheduled times of the day. The average pH on the teeth of intact rats before eating was in the neighbourhood of 8·0. After eating a cariogenic diet, or sugar alone, it rarely fell as low as 7·0. In sialoadenectomized animals, it was lower than in intact animals. It is concluded that in the rat dental caries may occur without the pH on the teeth falling to what is regarded by many investigators as the decalcification level for human teeth. The results of these experiments raise some questions concerning the acidogenic hypothesis of the aetiology of dental caries.

INTRODUCTION

MANY investigators seem to assume that localized acid decalcification of the enamel occurs as a result of bacterial enzymatic action on carbohydrates in the dental plaque and that this constitutes the initial lesion of dental caries. The validity of this assumption is difficult to prove or disprove.

It is believed by some that enamel is decalcified when the pH on the surface of the teeth falls to a level of 5·5 or below. FOSDICK and his associates (FOSDICK, CALANDRA, BLACKWELL and BURRILL, 1953; FOSDICK, 1956) have designated a pH of 6·0 and above as the safe zone; between 5·6 and 6·0 the questionable zone; and between 5·0 and 5·5 the danger zone. The pH of the plaque on human teeth may drop into the so-called danger zone after rinsing the mouth with a glucose solution (STEPHAN, 1944; STRALFORS, 1948) and also after eating various foods (HALDI and WYNN, 1955). These studies, however, provide no convincing evidence in support of the acidogenic hypothesis of the aetiology of dental caries.

As it is now possible to produce dental caries in the albino rat, it should be possible to determine, by frequent pH measurements on the tooth surface, whether and to what extent lowering of the pH on the tooth surface is a prerequisite to the initiation of dental caries in this animal. The present experiments were undertaken in the hope of shedding some light on this problem.

EXPERIMENTAL

A specially constructed antimony electrode, somewhat similar to the one described by STEPHAN (1940) was used for making the pH determinations. Small cylinders, approximately 10 mm in length, were made by drawing molten chemically pure

antimony metal into a glass tube with a diameter of approximately 1–2 mm. When the antimony had solidified, the glass tube was removed by tapping it gently with a small hammer. The cylindrically moulded antimony was soldered to a copper wire and then inserted into a small glass tube through which the copper wire had been drawn. The antimony was sealed in the end of the tube with wax. The tip, which was allowed to project 2–4 mm from the tube, was then polished with a fine Swiss file or with extremely fine sandpaper until it was 0·5 mm or less in diameter. The free end of the copper wire was connected with a Leeds–Northrup battery-operated pH meter. In the earlier experiments the calomel reference electrode was pressed on the rat's tongue, but it was later noted that the same pH readings were obtained when it rested on the nose, which is more readily accessible.

When applied to the tooth surface, the antimony electrode gives immediate and steady pH readings. The readings thus obtained are reproducible by removing the electrode, rinsing and drying it and again touching it on the tooth surface. Our experience with the antimony electrode has been different from that of CHARLTON (1956) who found it extremely difficult to produce two antimony electrodes which gave the same readings in the same buffers. The electrodes were tested before each experiment using pH 4·0 and pH 7·0 buffers. The electrodes must be stored in air and not in water. It has been our practice to repolish them as the tips become worn. The electrodes, tested each day with pH 4·0 and pH 7·0 buffers and on occasions several times a day, gave correct readings within 0·1 pH or less. We have been able to use the same electrode for hundreds of readings over a period of months.

Furthermore, CHARLTON found that while the glass electrode gave a stable reading when placed against the mucosa inside the mouth, the antimony electrode tended to give unstable readings. This instability was greatest when the electrodes were pressed against the tissues. Our antimony electrodes, on the other hand, gave stable readings when pressed against the oral mucosa and also when dipped into the saliva on the floor of the mouth.

As occlusal caries in the albino rat in our laboratories is initiated at the bottom of the fissures in the molar teeth, we were concerned lest the pH in these locations might be different from that on the surface of the tooth above and adjacent to the fissures. The fissures are so extremely narrow that we found it impossible to construct an electrode small enough to penetrate them. In order to obtain pH readings at the bottom of the fissure, we enlarged the fissure by drilling with a dental bur having a diameter slightly larger than the diameter of the electrode.

The technique of drilling the hole is a simple procedure as shown in Fig. 1. The animal is held with its back against the palm of the left hand of an attendant. The thumb and index finger are clasped snugly but not tightly around the animal's shoulder. The animal's right foreleg is held against its body by the attendant's left thumb and its left foreleg between the thumb and second finger. A loop at the end of a string about 20 in. long is caught over the upper incisors. The string is passed over the back of the left hand and between the fourth and fifth fingers and fastened by being wound several times around the latter. A loop at the other end of the string is caught over the lower incisors, passed down over the back of the thumb and

fastened by winding it around the little finger of the left hand. The mouth can then be opened by gently pulling apart the thumb and index finger. The operator pushes the tongue aside with a small tongue depressor while the attendant pushes back the buccal mucosa with a depressor. A head lamp and a head piece with magnifying glasses enable the operator to locate the desired fissure and to drill the hole without difficulty. The same procedure for holding the mouth open was followed in taking the pH readings.

As our experiments progressed, we became concerned lest the pH readings taken in the enlarged fissure might perhaps not be the true readings at the bottom of the fissure. We thought of the possibility that the side of the electrode might come into contact with the walls of the fissure and that if the pH at this location were different from that at the bottom of the fissure, the reading thus obtained might not be the accurate reading for the bottom of the fissure.

In order to resolve this problem, we coated one electrode with Duco cement leaving only approximately 1 mm of the tip exposed. With this arrangement, the antimony came into contact only with the bottom of the fissure. An experiment was then conducted on different days using fourteen rats as follows. The pH reading was taken with the point of the coated electrode touching the bottom of the enlarged fissure and immediately afterwards with an electrode that was not coated. The procedure was then reversed by taking the first reading with the uncoated electrode and the second reading with the coated electrode. The average of fifty-eight readings taken with the coated electrode was exactly the same as with the uncoated one. These results gave us confidence in the reliability of our procedure for obtaining pH measurements at the bottom of the enlarged fissure.

In later experiments we became interested in determining whether the pH at the bottom of the fissure is different from the pH on the occlusal surface of the tooth immediately adjacent to the fissure. Therefore we took numerous readings on this surface immediately before or immediately after taking the reading in the enlarged fissure. The average of a large number of readings on the tooth surface adjacent to the fissure was the same as the average of the readings with the electrode within the enlarged fissure. However, as we had followed the procedure in our earlier experiments of taking the pH reading in the enlarged fissure, and as it seemed possible that, under some circumstances, the pH might be different on the surface of the tooth and at the bottom of the fissure, it was decided to obtain the reading at the bottom of the fissure throughout all the experiments.

Experiments were done on both sialoadenectomized and on intact animals. The animals were sialoadenectomized by the procedure described elsewhere (HALDI, WYNN, SHAW and SOGNNAES, 1953). In the early experiments the animals were allowed to eat *ad libitum*. As it was desired in later experiments to obtain the pH readings immediately after eating and at specified intervals thereafter, the animals were trained to eat half their daily food allowance over a specified period in the morning and the remaining half over the same period of time in the afternoon. This procedure will be referred to as scheduled feeding. In the scheduled feeding experiments the first pH reading was taken about 9 o'clock in the morning before the

animals had eaten. Food cups were then placed in the cage, whereupon the animals would start eating immediately. At the end of 10 min, and in some experiments at the end of 20 min, the cup and watering tube were removed from the cage and the pH reading on the teeth taken without delay. The animal was then replaced in its cage and another reading taken 10 min later. In some experiments readings were taken again 20 and 30 min later. The feeding cups were then reintroduced into the cage and the animal allowed to eat for another 10 min in order to provide sufficient sustenance.

Experiments were conducted under a variety of conditions which will be described briefly under appropriate headings in the following section. The standard deviations are not given because we have found in numerous experiments, involving thousands of pH readings, that the standard deviation was always very close to 0·5.

RESULTS

pH *at different times of the day on teeth of sialoadenectomized rats allowed to eat* ad libitum. Shortly after the initiation of this study, we gained the impression that pH readings obtained at random when the animals were allowed to eat *ad libitum* were much more variable when taken in the afternoon than when taken in the morning. An experiment was then conducted to obtain more precise information on this point. Six sialoadenectomized rats were fed a cariogenic diet. After they had been on this diet for one day, pH readings were taken at intervals over a 30 day period at 9.00 a.m., 3.00 p.m., 8.00 p.m. and 9.00 p.m. Readings were taken on the first upper and lower molars in the right and left quadrants, giving a total of twenty-four readings at each specified time. The averages of the morning pH readings, as shown in Fig. 2, were
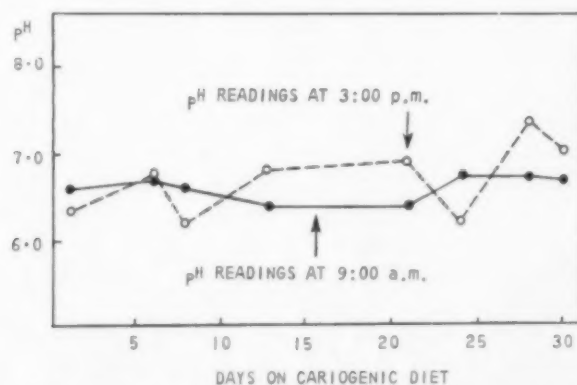
Fig. 2. pH in morning and afternoon on teeth of sialoadenectomized rats eating *ad libitum*.

fairly constant, ranging from 6·2 to 6·7, whereas the averages of the afternoon readings showed a pronounced irregularity, with a range from 6·2 to 7·5. We are unable to account for this irregularity in the readings taken in the afternoon. There was no

significant difference in the readings taken at 8.00 p.m. and 9.00 p.m. (Fig. 3). These readings were fairly constant. These results point to the importance of selecting the time of day when the readings are the least irregular, if pH determinations are to be made on the teeth of sialoadenectomized animals allowed to eat *ad libitum*.
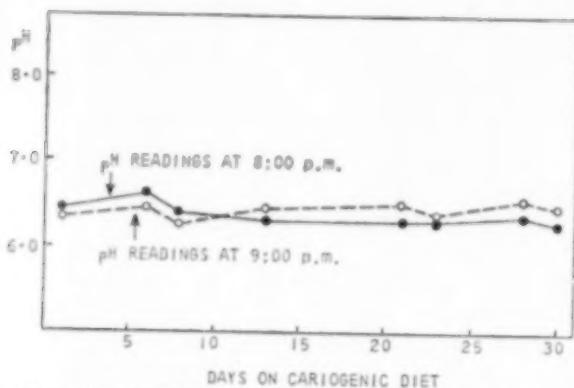
FIG. 3. pH readings at 8.00 p.m. and 9.00 p.m. on teeth of sialoadenectomized rats eating *ad libitum*.

pH *taken in the morning and afternoon on the teeth of sialoadenectomized rats on a scheduled feeding routine.* In the preceding experiments it was impossible to determine how much time had elapsed since the animal had last eaten. Although the rat normally consumes most of its food during the night, it will eat a little from time to time during the day if food is available. In this experiment twenty-four sialoadenectomized animals in individual cages were given half their usual food intake in the morning and the other half in the afternoon. They were allowed to eat for 1 hr, as we had found that our sialoadenectomized animals will not eat enough for sustenance in shorter feeding periods.

The pH readings were taken on the teeth of all the animals on different days 10 min after the feeding cups had been removed. The readings were taken at 9.00 a.m. and again at 3.00 p.m. the same day. The results are presented in Fig. 4. Each point on the curves is an average of ninety-six readings—one in each quadrant of each animal. It is apparent that, unlike the results obtained with *ad lib.* feeding, there was no appreciable difference in the average morning and afternoon readings.

pH *on teeth of sialoadenectomized and intact rats.* In our laboratories intact rats of our Wistar strain develop dental caries when fed a high-sucrose diet for a relatively long period. In order to accelerate the onset and progression of dental caries, our usual procedure is to sialoadenectomize the animals at weaning. This experiment was conducted to determine whether and to what extent the pH differs when taken on the teeth of intact and sialoadenectomized rats. Twelve animals were sialoadenectomized and their litter mates kept intact. All were allowed to eat *ad libitum*. Four pH readings were taken on each animal at 9.00 a.m.—one on the first molar of each quadrant—on different days over a 30 day period. The average pH readings on the

teeth of the sialoadenectomized rats were appreciably lower than in the intact animals (Fig. 5). The averages on the former ranged from 6·3 to 6·7 as compared with averages ranging from 7·7 to 8·0 in the latter. These pH readings on the teeth of intact rats were in the same range as those reported by THOMPSON and BRUDEVOLD (1954) and by JOHANSEN and ROGOSA (1957) on hamsters' teeth and by OCKERSE and DE JAGER (1957) on monkeys' teeth.
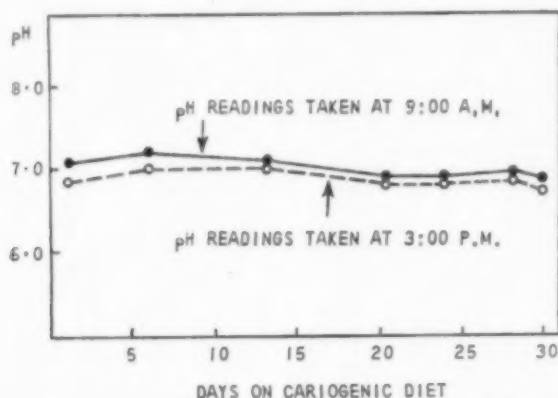
FIG. 4. pH readings at 9.00 a.m. and 3.00 p.m. on teeth of sialoadenectomized rats on scheduled feeding.

pH *on upper and lower molars of intact rats.* Our animals fed a cariogenic diet develop considerably more caries in the lower than in the upper molars. It was therefore considered of interest to determine whether there might be a difference in the pH of the upper and lower molars when the animals were fed this diet.
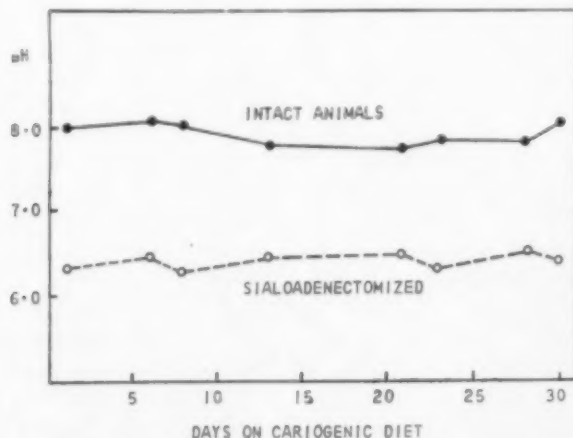
FIG. 5. pH on teeth of sialoadenectomized and intact rats.

Ten animals were placed on the diet at weaning and the first pH readings taken 30 days later. Readings were then taken at intervals over the next 28 days. On the specified days, four readings were taken on each animal—one each on the first molar in each quadrant. The food cups were removed from the cages on one morning and the following morning pH readings were taken before the animals were given anything to eat. The cups were then placed in the cages, the animals allowed to eat for 20 min and then the second pH readings taken on the same teeth. From the average readings presented in Fig. 6, it will be noted that there was no significant difference in the pH readings on the upper and lower molars either before or after eating. At the conclusion of the experiment, eleven small carious lesions were found in the lower and none in the upper molars.
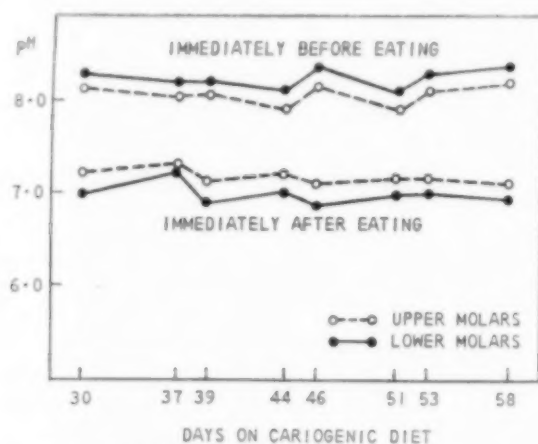
FIG. 6. pH on upper and lower molars of intact rats.

pH *on lower molars of intact rats immediately before and after eating a cariogenic diet and 10 min later.* This experiment was conducted on the lower molars of ten animals following the same procedure as in the preceding experiments, except that another reading was taken 10 min after eating. Readings were taken on the first molar in the right and in the left quadrant. The results are shown in Fig. 7. Within 10 min after eating there was a definite rise in the pH. On certain days another reading was taken 10 min and again 20 min later. Usually there was further rise in the pH at these times but it had not quite returned to the pre-eating level.

pH *on molars and dental caries experience of intact animals when sucrose was fed* ad libitum *and at scheduled times of the day.* Fifteen pairs of litter-mate albino rats were selected at the weaning age of 21 days and fed on a high sucrose cariogenic diet until they were 30 days old. From this time on the animals were fed by stomach tube three times a day all the constituents of a synthetic cariogenic diet except the sugar, which constituted 64 per cent of the diet. One animal of each pair was allowed to eat sugar *ad libitum* while its litter-mate was permitted to eat sugar in the morning

and again in the afternoon for 20 min at each feeding. pH readings were taken at intervals of approximately 2 weeks in the enlarged fissures of the lower right and left first molars. Readings were taken immediately before and immediately after eating and again 10 min later on the teeth of the animals on scheduled feeding. As the experiment was continued for 6 months, this gave a total of 24 readings on each animal before eating—a total of 360 readings on fifteen animals—and the same number immediately after eating and 10 min later. The pH readings also were taken every 2 weeks on the teeth of the animals that were allowed to eat sugar *ad libitum*, but there was no way of knowing when they had last eaten. At the conclusion of the experiment, the animals were sacrificed and the molars examined under a dissecting microscope and scored for caries.
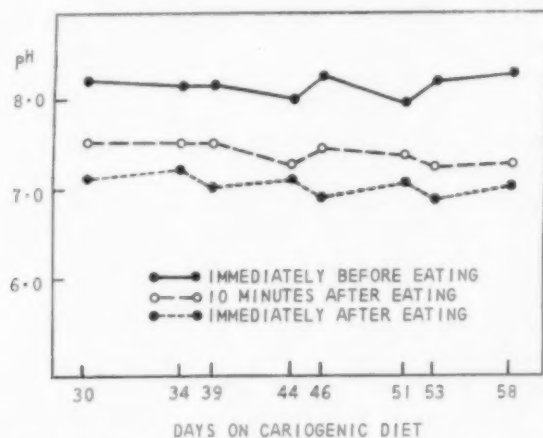
FIG. 7. pH on rats' teeth before and after eating.

The data on the pH readings and those on dental caries are given in Table 1. Out of all the pH readings that were taken, there were only eight below pH 7·0, the lowest of which was 6·7.

TABLE 1.   DENTAL CARIES AND pH ON TEETH OF INTACT ALBINO RATS FED
SUCROSE *ad libitum* AND AT SCHEDULED TIMES OF THE DAY

Experiment	No. of rats	Days on expt.	Ave. no. lesions	Ave. caries score	pH		
					Before eating	After eating	10 min later
Ad libitum	15	180	16	28	—*	—*	—*
Scheduled feeding	15	180	10	10	8·3	7·8	8·1

* As these animals were allowed to eat *ad libitum*, pH readings could not be taken with reference to the time of eating. The average of the pH readings taken at 9.30 a.m. was 8·0.

The animals on scheduled feeding had an insignificant amount of caries. There was an average of ten carious lesions in each animal, but these lesions were all extremely small. In the animals allowed to eat *ad libitum*, however, there was an appreciable amount of caries. The average number of lesions was larger than in their litter-mates on scheduled feeding and the lesions much more extensive. A number of these lesions were very far advanced.

DISCUSSION

A number of investigators have postulated a direct relationship between acid production from carbohydrates in the mouth and the initiation and the progress of dental caries (KESEL, 1956; MICHIGAN WORK SHOP, 1948; NATIONAL RESEARCH COUNCIL, 1952). This postulate is based on the assumption that the initial lesion of dental caries is produced by acid decalcification of enamel.

The idea that pH 5·0 is the critical level at which dental enamel is decalcified dates back to ENRIGHT, FRIESELL and TRESCHER (1932). These investigators found that, while enamel was etched when immersed in simple lactate and citrate buffer solutions ranging from pH 4·0 to pH 8·0, this did not occur when the buffers were saturated with tricalcium phosphate unless the pH was approximately 5·0 or lower. It was doubtless upon the basis of these findings, together with the fact that human saliva is believed to be saturated with tricalcium phosphate, that FOSDICK designated the range of pH 5·0 to pH 5·5 as the danger zone.

Later studies by BESIC (1953) would indicate that the "danger zone" should be established at a lower pH level. BESIC found that there was a total inhibition of any enamel change in solutions with maximal calcium and phosphate ion concentrations when the pH was as low as 3·5. Commenting on the report by ENRIGHT, FRIESELL and TRESCHER, he notes that their solutions did not have maximal calcium and phosphate ion concentration when the enamel was exposed to them, inasmuch as these solutions were acidified after saturation with $Ca_3(PO_4)_2$. The observations of HILLS and SULLIVAN (1958a) were substantially the same as those of BESIC. Enamel did not dissolve when the buffers were saturated with $Ca_3(PO_4)_2$ irrespective of whether the pH value was 7·0 or 4·0. When the buffers were not saturated, solution occurred; the amount and the pH level at which it occurred being related directly to the concentration of calcium and phosphorus in the buffer. When, however, powdered enamel was treated with saliva acidified with lactic acid to various pH values (HILLS and SULLIVAN, 1958b), the enamel usually began to dissolve at about pH 5·0 although there were indications that dissolution may have begun even at pH 6·0. ERICSSON (1949), on the other hand, concluded from his calculations that the critical pH point for enamel dissolution is generally between pH 5·5 and 6·5. The discovery of the increased solubility of calcium phosphate in the saliva in the physiological pH-region, he says, makes it clear that the saliva is not supersaturated to such a high degree as has hitherto been thought on the basis of the generally accepted solubility products for $Ca_3(PO_4)_2$ or $Ca_6(PO_4)_3OH$. These various observations leave as an open question the pH level at which tooth enamel might be decalcified in the

mouth *in vivo*. The highest level proposed by ERICSSON is pH 5·5–6·5, whereas there is strong evidence that it may be well below pH 5·0.

One would hesitate to apply without reservations these considerations on the saliva and tooth enamel of man to the saliva and tooth enamel of the rat. It should be noted, however, that the pH of the rat's saliva is of the same order of magnitude although somewhat higher than human saliva; also, the rat's saliva has a buffering capacity, as is shown by the fact that, after eating, the pH drops to a much lower level in sialoadenectomized than in intact animals.

Under the experimental conditions in the present study in which the lowest pH readings were obtained, namely, on sialoadenectomized animals allowed to eat *ad libitum*, it is possible that there may have been some acid decalcification of the enamel. However, in the experiments in which the salivary glands were intact, it would seem unreasonable to assume that decalcification would occur at the relatively high pH level observed after eating. Since dental caries develops under these feeding conditions, it may be concluded that a low pH on the surface of the teeth of the rat is not an essential prerequisite for the initiation of dental caries. While this does not disprove the acidogenic hypothesis of the aetiology of dental caries, it does offer some evidence in that direction. Similar evidence may be found in the studies of OCKERSE and DE JAGER (1957) who observed that the teeth of the vervet monkey may develop caries in an alkaline environment.

It is of interest to note that caries appeared much earlier and progressed much more rapidly in sialoadenectomized than in intact animals and that the pH on the teeth fell to considerably lower levels in the former than in the latter. It is possible that while the lower pH levels were not essential for the development and spread of dental caries, they may have provided a more favourable environment.

In a previous study (HALDI, WYNN, LAW, BENTLEY and RAMSAY, 1956) it was found that the spread of carious lesions in rats' teeth was associated with a lowering of the pH and an increase in the amount of lactate on the tooth. THOMPSON and BRUDEVOLD (1954) have reported pH readings on hamsters' teeth similar to ours on rats' teeth. The pH of tooth surfaces of the hamsters ranged from 7·4 to 8·5, whereas in a carious lesion, which was dried before the test, it was 6·7. Subsequently, JOHANSEN and ROGOSA (1957) reported similar findings on the hamster with respect to the pH on the tooth surface, no determinations of lactate having been made. They consistently found that active carious lesions gave lower pH values than sound surfaces. The mean pH on carious surfaces was 6·6 (range 5·5–7·7) as contrasted with pH 8·0 (range 7·2–8·8) on sound surfaces. The authors suggest that this difference points to the importance of an acid environment in the progression of established lesions. This, however, does not necessarily follow for, as we have pointed out previously (HALDI *et al.*, 1956), the progression of the carious lesion may conceivably provide a more favourable medium for the growth of acidogenic bacteria, in which case acid production may be incidental to and not the cause of the spread of the lesion.

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Fig. 1. Technique of enlarging the fissure in the rats' tooth.

PLATE 1

THE ORGANIZATION OF THE PERIODONTAL MEMBRANE FIBRES OF THE DEVELOPING MOLARS OF RATS

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Abstract—Rats, 15–35 days old, were used to study the formation and organization of the periodontal membrane fibres of the molar teeth. Celloidin sections were prepared and stained by differential stains for connective tissue fibres. In the early development of the periodontal membrane there is a definite oblique orientation of the fibroblasts towards the gingival region. At the same time fine collagenous fibres are observed arising from the cementum and running parallel to the cells. These fibres appear to be entrapped by the forming alveolar bone. As the root continues in development, the fibroblasts as well as the fibres maintain this fundamental relationship. As the tooth erupts into the oral cavity and enters clinical occlusion with its opposing tooth the attachment fibres become organized into the functional six groups as usually described. It appears that the stresses of functional occlusion cause a thickening of the fibres.

INTRODUCTION

The arrangement of the principal fibres of the periodontal membrane into the six functional groups was first classified by Black (1887) and since that time this classical description has been generally accepted in textbooks of oral histology and periodontia. It has also been agreed to that the arrangements of these fibres is a functional adaptation to sustain the tooth against all the forces to which it is subjected such as mastication, occlusal stress and lateral pressure.

On the other hand there has been a distinct lack of study concerning the development and organization of these attachment fibres during the pre-functional period. Thus it was felt that a detailed systematic investigation of the formation and organization of the supporting structures of the tooth from the pre-eruptive stage to functional occlusion was desirable.

The rat was chosen for this investigation primarily through the necessity of having available material exhibiting consecutive developmental and eruptive patterns, a situation which would be difficult to attain with human material. In addition, the molar teeth of the rat are of limited growth and are similar in both development and eruption to the human primary teeth.

MATERIALS AND METHODS

The teeth and jaws used in this study were from rats ranging in age from 15–35 days. At the time of sacrifice the heads were removed and immediately fixed in an alcoholic acetic acid formalin solution for at least 4 days. The specimens were

decalcified with a solution of 10% nitric acid in 10% formalin. After decalcification
they were dehydrated and infiltrated in the routine manner for nitrocellulose embed-
ding. This was achieved by embedding blocks of tissue in 50% nitrocellulose which
were then sectioned at 20–35 μ thickness. The specimens containing the complete head
were cut so that mesiodistal sections were prepared. Alternate sections were stained
with Mallory's connective tissue stain, by the periodic acid Schiff reaction, and by
Pearson's silver gelatin impregnation technique.

RESULTS

Fifteen-day old rat

Mesial surface. In a 15-day old rat the mesial cusp of the upper first molar is
directly under the oral mucosa. The bifurcation and development of the roots are
well advanced. The crest of the mesial alveolar bone is located in the region of the
upper half of the crown (Fig. 1). The alveolar bone consists of anastomosing osteo-
genic spicules which are surrounded by clusters of osteoblasts (Fig. 2). In the region
of the cemento–enamel junction, fine collagenous fibres arising from the cementum
course occlusally, lying directly underneath the enamel epithelial layer. Below this
region, fibres pass from the cementum upward and obliquely towards the osteogenic
spicules. As these fibres reach the bony spicules, some become condensed into the
bony matrix while others penetrate the osseous material to terminate in the osteo-
blastic masses. In the apical third of the developing root, the fibroblasts are arranged
in an oblique occlusal direction towards the bone spicules (Fig. 2).

Interproximal area. The periodontal membrane of the distal surface of the upper
first molar is further advanced in development than the mesial surface of the adjacent
second molar (Fig. 3). The crest of the interdental bone is located above the cemento–
enamel junction of both teeth (Fig. 3). The interdental bone consists of thin
anastomosing osteogenic trabeculae with a wide trabecula-free spongiosa. In the
developing periodontal membrane of the distal surface of the upper first molar,
collagenous fibres are seen arising from the cemento–enamel junction and passing
occlusally directly underneath the enamel organ. These will become the free gingival
fibres. Below this landmark all the remaining demonstrable attachment fibres are
passing in an oblique occlusal direction to become embedded in the developing bone.
In contrast, there is no indication of thick bands of collagenous fibres in the mesial
periodontal membrane of the second molar. However, delicate fine fibres may be
observed arising in the region of the cemento–enamel junction to pass occlusally.
Below this junction the fibroblasts are orientated in an oblique gingival direction
from the cementum towards the adjacent bone.

Twenty-one-day old rat

Mesial surface. By the twenty-first day the upper first molar has erupted into the
oral cavity (Fig. 4). The mesial alveolar crest has been lowered to the apical third
of the crown. The alveolar bone has become thicker and is composed of a con-
tinuous plate of bone (Figs. 4 and 5). There has been no further organization of the
free gingival fibres. The remaining apically arranged fibres are still orientated in the

occlusally oblique direction. However, the angle of the obliqueness is less than at the fifteenth day.

Interproximal area. In the interproximal area the position of the crest of the interseptal bone is dependent upon the specific tooth surface. In relation to the first molar the crest of the bone is below the cemento–enamel junction, while it is still situated on the crown area of the second molar (Figs. 4 and 6). The interseptal bone now consists of thick anastomosing trabeculae. In the developing periodontal membrane of the distal surface of the upper first molar the future free gingival fibres have become thicker in appearance. Below the cemento–enamel junction a wide band of fibres courses downward to become inserted into the bone, and immediately underneath, a horizontally arranged group of fibres is visible. Apical to this group, the fibres are arranged in the oblique direction passing upward from the cementum to the bone. In the adjacent mesial periodontal surface of the second molar, fine free gingival fibres are now demonstrable. Here also the remaining fine fibres are directed upward in the oblique direction to become condensed in the forming bone (Fig. 6).

Twenty-five-day old rat

Mesial surface. At 25 days of age, the upper first molar has erupted into functional occlusion. There has been a further deposition of parallel lamellae to the alveolar bone. The crest of the bone is now located at the region of the cemento–enamel junction (Figs. 7 and 9). The free gingival fibres have become thicker and appear to radiate into the lamina propria of the gingiva and, at the same time, fine collagenous fibres are observed in the connective tissue papillae of the lamina propria. The supracrestal fibres of the free gingival groups are easily demonstrable, arising from the cementum and coursing over the crest of the bone. In the occlusal third of the alveolar bone fibres are arranged in a horizontal fashion passing from the cementum to be inserted in the bone. Below this boundary and continuing apically the attachment fibres are still directed upwards and obliquely from the cementum to the adjacent bone (Fig. 8).

Interproximal area. The crest of the interdental bone is lowered to the occlusal third of the root (Figs. 7 and 9). With continual deposition of bone, the occlusal third of the interdental bone is dense in appearance. Below the cemento–enamel junction fibres from the mesial surface of the second molar and distal surface of the first molar pass across the crest to interdigitate in the midregion. A few fibres in this region are seen passing downward to become inserted in the crestal bone. In the region of the occlusal third of the bone, fibres originating from the cementum course downward to be inserted in the alveolar bone. The fibres below this region are still arranged in the oblique direction.

Thirty-five-day old rat

Mesial surface. By the thirty-fifth day the alveolar crest of the mesial alveolar bone has been lowered to the occlusal third of the root (Figs. 10 and 11). With the addition of the 10 days of functional occlusion, the alveolar bone has become denser

in appearance. The free gingival fibres are thicker and radiate into the connective tissue papillae of the gingivae. With the lowering of the alveolar crest to its physiological location the previously described horizontal fibres now become the alveolar crest fibres, and the previously described oblique fibres are organized in a horizontal position. Below this region the fibres remain oblique and apical fibres respectively (Fig. 11).

Interproximal area. At this time sequence, the trans-septal fibres have become very thick so that it appears that they originate in the cementum of one tooth and pass across the interproximal space to become inserted in the cementum of the adjacent tooth (Fig. 12). The fibres in the region of the occlusal third of the bone appear as the alveolar crest group, whereas the remaining apically arranged fibres are seen passing in an oblique direction.

DISCUSSION

The fact that the principal collagenous fibres of the periodontal membrane are arranged into six functional groups has been well established by many investigators. However, there is a lack of knowledge concerning the development and organization of these attachment fibres into the functional groups. The infrequent reports are probably due to the difficulty in obtaining adequate human material to enable one to follow the development and arrangement of the fibres in successive stages of tooth development and finally clinical eruption. In the rat not only can one easily obtain specimens of progressive stages of tooth formation to clinical occlusion, but the size of the animal is such that one can section the intact head and study the comparative development of the three molar teeth and their periodontium in one section.

ORBAN (1957) and NOYES, SCHOUR and NOYES (1955) state that, at the beginning of active eruption of the tooth and at the beginning of cementum and alveolar bone formation, three layers of fibres may be differentiated in the forming periodontal membrane. These are alveolar fibres and a plexus intermedius of unorientated fibres. These fibres persist until the tooth erupts into the oral cavity and comes into clinical occlusion. At this time these fibres are replaced by the principal fibres, and the fully developed character of the periodontal membrane is reached.

In the present investigation a difference was noted from the above description. In the developing molar teeth of the rat there was no indication of three different layers in the forming periodontal membrane. The formation and organization of the attachment fibres begins as early as the first appearance of cementum on the root. The first indication of fibre formation is the orientation of the fibroblasts in a gingival oblique direction towards the forming bone spicules.

Later, fine fibres may be seen arising from the cementum and passing towards the bone. These fibres become inserted either in the developing bone matrix or in the cluster of osteoblasts. At a further developmental stage these fibres become thicker and are easily demonstrable either by Mallory's connective tissue stain or by silver impregnation.

The arrangement of these fibres into the six functional groups is related to (1) the stage of eruption and (2) the height of the alveolar bone. Before the tooth has erupted

into functional occlusion, the crest of the alveolar bone is located above the cemento–enamel junction. As long as the crest is above the cemento–enamel junction, all the demonstrable fibres except the free gingival group are orientated in an oblique direction. However, as the tooth erupts into clinical occlusion and the crest of the alveolar bone is lowered to the cemento–enamel junction, the oblique fibres found at the upper third of the bone straighten out to become horizontal. When the alveolar bone has reached its adult physiological position, that is, at the upper third of the root, these horizontal fibres become the alveolar crest fibres and the fibre groups immediately below are now arranged in an horizontal direction.

The trans-septal fibres are described in the textbooks as springing from the cementum of one tooth and passing across the interproximal space to be attached to the cementum of the approximating tooth. In the present study it was noted that these fibres did not become organized until both approximating teeth are in clinical occlusion. At this stage fibres from one tooth pass towards the middle of the interproximal space to interdigitate with the fibres arising from the cementum of the adjacent tooth. After the teeth are in functional occlusion, these fibres become thicker, and the interlacing fibres appeared to become cemented at the mid-line, giving the appearance that these fibres pass from one tooth to another.

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Key to abbreviations

A.B.   alveolar bone
A.C.   alveolar crest
C.E.   cemento–enamel junction
D.   dentine
E.   enamel
F.G.   free gingival fibres
H.   horizontal fibres
O.   oblique fibres
Pu.   pulp
1M.   first molar
2M.   second molar

All sections were stained with Mallory's connective tissue stain.

FIG. 1. Fifteen-day old rat. Upper first and second molar. Note the position of the teeth as well as the alveolar crests of both the mesial and interproximal surfaces. × 13.

FIG. 2. Fifteen-day old rat. Mesial surface of upper first molar. Note the oblique orientation of the fibroblasts and fibres springing from the cementum and inserting into the developing bone spicules. × 38.

FIG. 3. Fifteen-day old rat. Interproximal surface of upper first and second molar. Note the obliquely arranged fibres in the distal surface of the first molar and the absence of demonstrable fibres in the mesial surface of the second molar. × 38.

FIG. 4. Twenty-one-day old rat. Note the eruption of the first molar and the position of the second molar. × 13.

FIG. 5. Twenty-one-day old rat. Mesial surface of first molar. The alveolar crest of the bone is still above the crown and the fibres of the periodontal membrane are still oblique in direction. × 38.

FIG. 6. Twenty-one-day old rat. Interproximal surface of upper first and second molars. Note the appearance of the free gingival, alveolar crest horizontal and oblique fibres of the distal surface of the first molar and only oblique fibres are present in the mesial surface of the second molar. × 38.
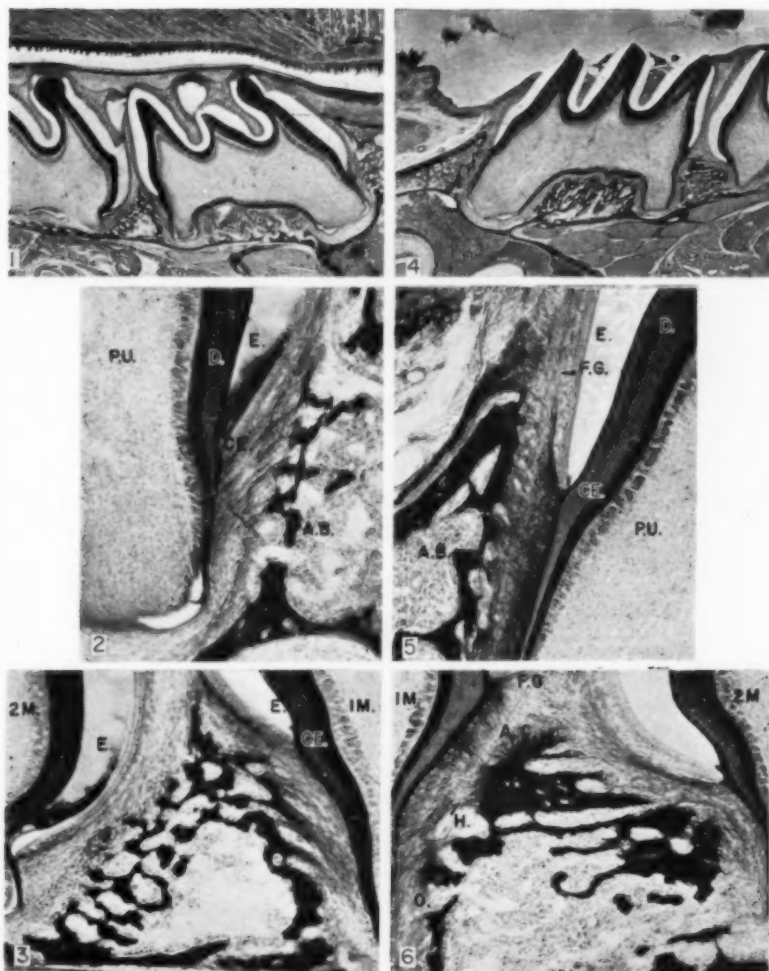
PLATE 1

Plate 2
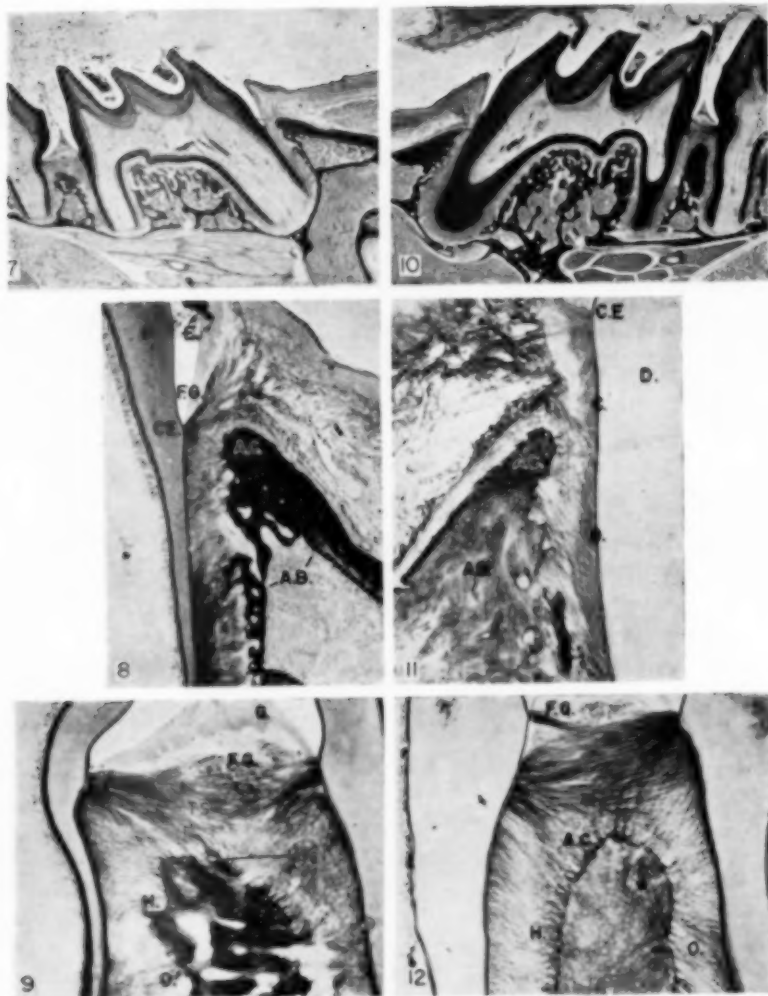
Key to abbreviations

A.B.	alveolar bone
A.C.	alveolar crest
C.	cementum
C.E.	cemento–enamel junction
D.	dentine
E.	enamel
F.G.	free gingival fibres
G.	gingiva
H.	horizontal fibres
O.	oblique fibres
TS.	trans-septal fibres

All sections were stained with Mallory's connective tissue stain.

Fig. 7. Twenty-five-day old rat. Upper first and second molar. The teeth are in functional occlusion. Note the position of the alveolar crests of the bone on the mesial and interproximal surfaces. ×13.

Fig. 8. Twenty-five-day old rat. Mesial surface of the first molar. The alveolar crest is at the cemento–enamel junction. Note the presence of free gingival fibres, horizontal and oblique fibres. ×38.

Fig. 9. Twenty-five-day old rat. Interproximal surface of the upper first and second molars. Note the attachment fibres arising from the cementum of both teeth coursing towards the midline, and interlacing. ×38.

Fig. 10. Thirty-five-day old rat. Upper first and second molars. Note the dense appearance of the bone. ×13.

Fig. 11. Thirty-five-day old rat. Mesial surface of the first molar. The alveolar crest is now found below the cemento–enamel junction. All the functional fibre groups can now be seen. ×38.

Fig. 12. Thirty-five-day old rat. Interproximal surface of an upper first and second molar. Note the thick trans-septal fibres as well as the remaining fibre groups. ×38.

AN IMPROVED SILVER METHOD FOR STAINING NERVE FIBRES IN DECALCIFIED SECTIONS OF TEETH

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Abstract—A silver staining method is described in which dewaxed sections of decalcified teeth are impregnated in a saturated solution of silver cyanate in dilute collidine buffer, pH 7·3, and developed in a buffered hydroquinone-sulphite solution. The impregnation-development process is repeated three or four times until a satisfactory intensity of staining is obtained. Non-myelinated nerve fibres are specifically stained black in a pale yellow background.

INTRODUCTION

THE controversy which has surrounded the innervation of human dentine for so long has been due mainly to the absence of a reliable and specific staining technique which can differentiate the fine terminal nerve fibres in the dental pulp and adjacent dentine. Only a few of the many available silver impregnation methods can be regarded as specific and none can be relied upon to produce consistently uniform results.

Following an investigation (ROWLES and BRAIN, 1956; ROWLES, 1960) of the silver staining method of UNGEWITTER (1951) we have examined the possibility of using a saturated solution of silver cyanate as impregnating solution.

In preliminary experiments, nerve staining was obtained after a single impregnation for 1 hr in silver cyanate solutions having pH 5·5–8. The most intense staining occurred at pH 8, but above pH 7 the staining of other tissue elements became increasingly evident.

According to the theory of silver staining developed by HOLMES (1943), SAMUEL (1953a, b) and PETERS (1955a, b, c) discrete staining is due to the formation of silver nuclei in certain tissue elements during impregnation. The bulk of the silver is taken up by the section as silver ions in loosely bound form (reducible silver) and, during reduction with a suitable developer, this is deposited upon the silver nuclei, giving the final visible stain. Below pH 8 the formation of silver nuclei occurs most rapidly in the nerve fibres. Therefore, in order to minimize the staining of the non-nervous structures the time of impregnation must be kept as short as possible.

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PETERS (1955a) found that the uptake of reducible silver is practically complete in 10 min at 37°C. However, in such short periods the number of nuclei formed is insufficient to produce a satisfactory intensity of staining upon development. Prolonged impregnation increases the number of silver nuclei but also their distribution so that the specificity and eventually the intensity of nerve staining decreases.

This difficulty can be overcome by the use of the repeated impregnation and development technique utilized by UNGEWITTER. After a short impregnation and development virtually all the silver is contained within the nerve fibres and this forms nuclei for further deposition of silver during subsequent development. The effect of any silver nuclei formed during the second and subsequent impregnations is negligible compared with that of the silver deposits already present. Three or four such treatments are generally sufficient to produce a suitable intensity of staining.

METHOD

Impregnation is carried out in a saturated solution of silver cyanate in 0·005 M collidine—nitric buffer at pH 7·3 after a brief forebath treatment of the sections in the dilute buffer alone. A hydroquinone–sulphite developer is used which contains borax to stabilize the pH at a level where the development process was found to be most efficient.

The method has been applied principally to sections of teeth fixed in 10% formalin in physiological saline, decalcified in 10% formic acid and embedded by the diethylene glycol distearate method described by BRAIN (1955). It has also been applied successfully to sections embedded in paraffin wax.

Reagents required
    Sodium cyanate (pure), 5% wt./vol. solution, freshly prepared.
    Silver nitrate (AnalaR), 12·5% wt./vol. solution.
    sym-Collidine (redistilled).
    Nitric acid (pure), 2 N solution.
    Acetic acid, 10% wt./vol. solution.
    Sodium sulphite (AnalaR), 40% wt./vol. solution.
    Hydroquinone, 2% wt./vol. solution, freshly prepared.
    Borax (sodium tetraborate, $Na_2B_4O_7.10H_2O$) (AnalaR), 38 g dissolved in water and made up
        to 1 l. (0·1 M).
    Ethanol, absolute.
    Brom-cresol purple solution, 0·04%.
    Phenol red solution, 0·02%.

Preparation of silver cyanate
    Silver nitrate solution, 200 ml, was run slowly from a separating funnel into an equal volume of sodium cyanate solution with constant stirring during the precipitation. Traces of carbonate were removed from the resulting mixture by the addition, drop by drop, of 10% acetic acid until a drop of the supernatant solution produced a yellow colour with brom-cresol purple indicator.
    The precipitate was filtered on a sintered glass Buchner funnel and then washed three times with 100 ml portions of cold distilled water, and three times with 100 ml portions of ethanol, the precipitate being stirred on the filter to ensure complete mixing with the wash liquid. The funnel was then covered with black paper to protect the silver cyanate from light and dust and sucked dry at a filter pump.
    The preparation was carried out in diffused light and the product stored in a dark glass bottle.

Collidine buffer solution, 0·1 M

Collidine, 13·0 ml, was shaken with 800 ml distilled water* and 2 N nitric acid run in from a burette until the pH of the mixture was 7·4 (approximately 20 ml 2 N acid). The pH was measured either with a pH meter or with phenol red indicator (by comparison of the colour with standards) using small portions of the solution. After adjustment of the pH the volume of the solution was made up to 1 litre. The solution was stored in a dark glass bottle.

* A brown oily impurity which separates from some commercial collidine specimens is adequately removed by filtration through several layers of filter paper.

Preparation of solutions for use

Forebaths. One volume of collidine buffer is diluted with 19 volumes of distilled water.

Impregnating solutions. Silver cyanate powder, 25–30 mg, is suspended in 5 ml distilled water by shaking vigorously in a glass stoppered tube. Collidine buffer, 2 ml, and distilled water to 40 ml are added.

Developing solution. A mixture of 10 ml sodium sulphite solution, 25 ml borax solution and 5 ml hydroquinone solution is diluted to 100 ml with distilled water.

Staining technique

(1) Place the forebaths (2) and the impregnating solution in an air oven at 60°C. (It is advisable to warm the solutions first, in order to attain the working temperature rapidly).

(2) Remove the wax from the sections by passing them through benzene (two changes), ethanol (two changes), 50% ethanol, to distilled water.

(3) Immerse sections in first forebath for 5 min at 60°C.

(4) Transfer to the second forebath for 5 min at 60°C.

(5) Transfer to the impregnating bath for 5 min at 60°C.

(6) Wash in distilled water at room temperature for 2 min.

(7) Place in developing solution for 1–3 min at room temperature and gently agitate the slide to ensure even development of the section.

(8) Wash well in distilled water to remove traces of developer.

(9) Repeat stages (3)–(8) until the nerve staining appears to be satisfactory when examined under the microscope (three to four applications are generally sufficient).

(10) Deyhdrate in 50% ethanol, then ethanol (two changes), clear in benzene and mount in Canada balsam.

RESULTS

The staining of nerve fibres, by this method, in part of a distearate embedded tooth section is illustrated in Fig. 1. Non-myelinated nerve fibres are stained black in a pale yellow background. Myelinated nerve fibres appear brown. Although sufficient background staining is usually present, counterstaining the section with haematoxylin allows the nerve fibres to be orientated in relation to the surrounding tissues, and particularly at the junction of pulp and dentine (Figs. 2a and 2b). Toning of the sections has not been found necessary.

DISCUSSION

The silver cyanate impregnating solutions used in this method give a silver concentration of 0·02% Ag at 60°C, the silver ion concentration being somewhat smaller owing to a partial reaction with the collidine, while the pH (measured at room temperature) is 7·3. The conditions of impregnation therefore fall between those used in methods of the type introduced by HOLMES (1943), which employ very dilute silver solutions (0·006–0·0006% Ag) at high pH (8–9), and those used in the methods of PEARSON and O'NEILL (1946), 1·2% Ag at pH 4·4, and UNGEWITTER (1951), 0·6% Ag at pH about 6. The conditions are most similar to FEARNHEAD and LINDER'S (1956) modification of HOLMES's method which uses 0·006% Ag at pH 7·0.

Apart from affording a convenient method of preparing an impregnating solution of suitable composition, the use of a saturated solution of silver cyanate may have other advantages. In our experiments with urea-containing solutions, we observed, under some conditions, an apparent catalytic effect on the impregnation in the presence of an excess of solid silver cyanate. Although it has not been possible to demonstrate a similar effect with the simple silver cyanate solutions, the staining results appear on the whole better than those obtained with comparable silver nitrate solutions. An analogous observation was made by ROMANES (1950) who found that better results were obtained by impregnation in a saturated solution of silver chloride in ammonia if excess solid was present. In routine use the presence of excess solid may also minimize the effect on the silver concentration of accidental impurities, or such phenomena as adsorption on the vessel walls or dust particles which often occur with very dilute solutions of metal ions.

It has not been found necessary to use double glass distilled water. The normal laboratory distilled water supply obtained from a copper still gives similar results to those obtained with more purified samples.

Few satisfactory buffers are available for silver staining in this pH range. Borax–boric acid buffers have very little buffering power at pH values below 7·5 (FEARNHEAD and LINDER (1956) found it necessary to use full strength buffer (0·05 M) to maintain the pH at 7·0). Collidine buffers, however, cover a pH range from 6·5–8·0 and therefore exert a maximum buffering at the pH of the impregnating solution.

The difficulty of obtaining specific staining of nerve fibres of suitable intensity, mentioned earlier, can also be overcome by the use of physical development. In this process, after removal of the reducible silver from the section, silver from the developer is deposited upon the silver nuclei in the section until the desired degree of staining is obtained. Such developers, however, are notoriously capricious in their action. Even the improved developers recommended by PETERS (1955b) and FEARNHEAD and LINDER (1956) are unstable and require critical timing to obtain the best results. We have preferred to use a more stable chemical developer together with the technique of repeated impregnation and development by means of which the intensity can be increased as required without altering the specificity of the staining.

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FIG. 1. A longitudinal section from a human premolar tooth. ×60.
A portion of the coronal pulp (*P*) showing numerous nerve fibres. *D* = Dentine.

FIG. 2a. A section of pulp and dentine *in situ* from a human canine tooth. ×600.
Non-myelinated nerve fibres are seen passing between the odontoblasts (*O*) into the dentine. *D* = Dentine. *PD* = Predentine.

FIG. 2b. The same section and field as Fig. 2a. Counterstained with Harris's haematoxylin to contrast the nuclear structures.

PLATE 1
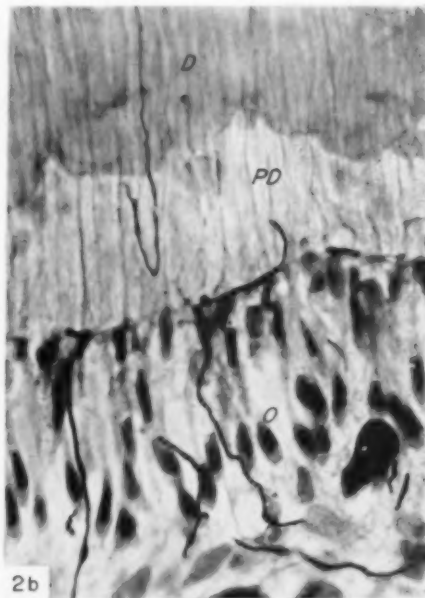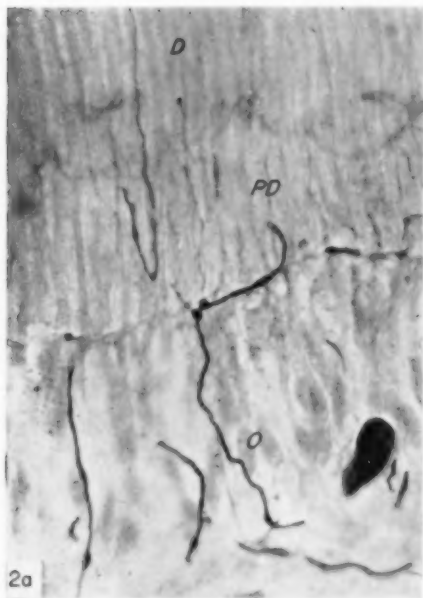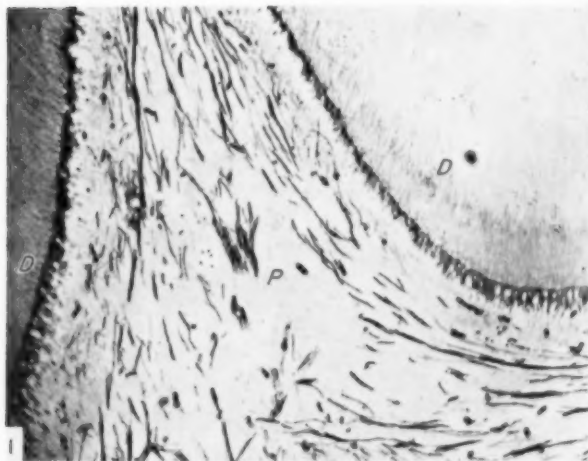
CARBOHYDRATE MATERIAL IN CARIOUS DENTINE—I

PRELIMINARY INVESTIGATIONS

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Abstract—Carious dentine hydrolysates, prepared using strong mineral acid (6 N HCl), are brown and contain insoluble black material. However, hydrolysates of carious dentine prepared using 1·5 N sulphuric acid, 5 N formic acid, 2 N sodium hydroxide and collagenase, though light brown, were devoid of any black precipitate. Autoclaving dissolved 70 per cent of carious dentine, and the resultant light brown solution was also free of insoluble black material. These results indicated that the precipitate in strong mineral acid hydrolysates of carious dentine probably formed during the hydrolysis procedure. The possibility that this feature was due to the presence of carbohydrate material was supported by the results of carbohydrate analyses. It was found that carious dentine contained approximately 4 per cent "glucose" units of carbohydrate, in contrast to sound dentine which contained 0·4 per cent "glucose" units. Carious dentine contained material giving a positive Elson–Morgan reaction, but paper chromatographic studies failed to indicate the presence of the amino-sugars glucosamine or galactosamine. The fractions of carious dentine resistant to collagenase action or autoclaving contained higher concentrations of carbohydrate material.

INTRODUCTION

DURING investigations into the amino acid composition of carious and sound dentine (ARMSTRONG, 1959), it was observed that acid hydrolysates of the organic matrix of carious dentine were markedly brown, and contained a brownish-black precipitate. Quantities as small as 5 mg of carious dentine matrix showed this effect, whereas a comparable sound dentine hydrolysate was colourless and free from any precipitate.

This phenomenon was observed by DEAKINS (1941) who suggested that the black precipitate might be a melanin-like pigment which had been formed in the matrix during caries. The acid hydrolysis brought about the dissolution of the matrix, and released the insoluble pigment particles which settled out as a black precipitate. DEAKINS also considered the possibility that the black substance may have been formed during the hydrolysis.

In a series of papers by DREIZEN and his co-workers (1949, 1950, 1957, 1958) it was shown that the demineralized matrix of sound dentine would react, *in vitro*, with certain glucose fermentation intermediates and degradation products. The resultant dentine preparations were brown and on acid hydrolysis a black precipitate was obtained. It was suggested that the brown pigmentation associated with dental caries might be the result of reaction between the dentine matrix and carbohydrate degradation products.

Although the black precipitate found in acid hydrolysates of carious dentine might be due to the release of particles present in the matrix, there is the alternative possibility that the material was actually formed during the acid hydrolysis. It is known that acid hydrolysis of proteins sometimes results in the formation of a black or brownish-black precipitate called humin (or "melanin"), and this effect is very marked in carbohydrate-containing proteins.

Using a histochemical method, ENGEL (1950) found that carious dentine gave a positive reaction for polysaccharide, and suggested that this was due to an unmasking of dentine ground substance by a depolymerization reaction which occurred during caries. Apparently he did not consider the possibility that the result might also be accounted for in terms of reaction or contamination of the dentine with carbohydrate material of extrinsic origin.

An investigation was, therefore, undertaken to determine whether significant amounts of carbohydrate material could be detected in carious dentine.

MATERIAL AND METHODS

Sound dentine (SD)

Sound dentine particles were prepared as previously described (ARMSTRONG, 1958) with the exception that the disintegration was carried out using a vertically oscillating ball mill (Glen Creston microdisintegrator).

Carious dentine (CD)

Carious dentine was collected as described previously (ARMSTRONG, 1958) and the disintegration effected with a Glen Creston microdisintegrator.

Demineralization

The EDTA method previously described (ARMSTRONG, 1958) was employed.

Partial hydrolysates of sound and carious dentine

Partial acid hydrolysates of the demineralized sound and carious dentine powders were made using either 5 N formic acid or 1·5 N sulphuric acid. For the formic acid hydrolysis approximately 10 mg quantities of the dentine powders were accurately weighed out into test-tubes and 10 ml of the formic acid solution added. The tubes were sealed and placed in a boiling water bath for several hours to effect solution of the particles.

For the sulphuric acid hydrolyses 10 mg quantities were weighed into test-tubes and 5 ml of 1·5 N H_2SO_4 was added. The tubes were capped with glass stoppers and placed in a boiling water bath for 30 min, with occasional shaking.

Collagenase hydrolysates of sound and carious dentine

Demineralized sound and carious dentine particles, 10 mg quantities, were treated with 5 ml of a 50 mg% solution of collagenase, using the method previously described (ARMSTRONG, 1958). The collagenase used was a partially purified preparation, supplied by the Wellcome Research Laboratories, and contained approximately 22 Q

units of collagenase/mg. Fuller information is given in the reference quoted. The incubation at 37°C was continued overnight with the addition of thymol crystals to inhibit microbial growth. Approximately 75 per cent of the carious dentine matrix and 95 per cent of the sound dentine matrix went into solution as a result of collagenase action.

Autoclaving of sound and carious dentine

Demineralized sound and carious dentine, 10 mg quantities, were placed in tubes, and 5 ml of water added to each. The suspensions were autoclaved for 1 hr at 15 lb/in², and then removed and shaken. The autoclaving procedure was repeated twice. Approximately 70 per cent of the carious dentine matrix and 90 per cent of the sound dentine matrix was brought into solution by this procedure.

Carbohydrate determinations

The first three methods of analysis described are fully discussed by DISCHE (1955). The hexosamine method employed was that described by LAURELL (1958).

(1) α-*Naphthol method.* Of the solution under test, 0·5 ml is pipetted into a boiling tube. Of 89 volume per cent H_2SO_4, 4·5 ml is added with cooling in an ice bath. After 1 min the mixture is shaken in ice and then transferred to a water bath at room temperature. The tube is then placed in a boiling water bath for 3 min and finally cooled in tap water.

Of a freshly prepared 2% alcoholic solution of α-naphthol, 0·2 ml is added and the tube shaken. A standard glucose solution and reagent blank are run at the same time. After standing for 1 hr, the colour developed is read at 550 mμ.

(2) *Anthrone method.* A 2% solution of anthrone in concentrated sulphuric acid is prepared and cooled to 10–15°C in an ice bath. Of the solution under test, 5 ml is carefully layered over 10 ml of this anthrone solution and allowed to cool in the bath. The mixture is shaken in the ice bath and then removed and brought to room temperature. It is then placed in the boiling water bath for 16 min, cooled, and the final colour is read at 625 mμ. A glucose standard and reagent blank are run simultaneously.

(3) *Cysteine method.* Of a mixture of six volumes of concentrated H_2SO_4 plus one volume of water, 4·5 ml is added to 1 ml of the test solution which is cooled in ice-water. After 1 min the mixture is shaken, then transferred to tap-water for 2 min and to a boiling water bath for 3 min. After cooling 0·1 ml of a 3% solution of cysteine hydrochloride is added and the mixture shaken. In a few minutes an intense yellow colour develops and this is read within the hour at 413 mμ. The glucose standard and reagent blank are similarly treated.

(4) *Hexosamine* (*amino-sugar*) *determination.* Of the demineralized dentine powder under examination, 10 mg is weighed into a 5 ml ampoule and 1 ml of water added, followed by 1 ml of 2 N HCl. The ampoule is sealed and placed in an oven at 100°C for 16 hr. The ampoule is then opened and the contents brought to neutrality by titration with 4 N NaOH, using a drop of 0·5% phenolphthalein as an indicator. When the indicator turns pink the solution is made slightly acid by the addition of

a few drops of dilute HCl. The solution is quantitatively transferred to a 5 ml volumetric flask and made up to 5 ml with water. Of this solution, 1 ml is pipetted into an ampoule and 1 ml of freshly prepared acetylacetone reagent added (1·5 ml redistilled acetylacetone dissolved in 50 ml of 1·25 N Na_2CO_3). The ampoule is sealed and placed in a thermostatically controlled water bath at 92°C (±0·5°C) for 45 min. Of 96% ethanol, 5 ml is pipetted into a test-tube and cooled in an ice bath. The ampoule is cooled in tap-water, opened, and 1 ml of the contents pipetted into the ethanol. Of cold Ehrlich's reagent (800 mg p-dimethyl-aminobenzaldehyde, 15 ml concentrated HCl and 15 ml ethanol), 0·5 ml is added, and the contents of the tube mixed. The tube is then placed in a bath at 60°C for 10 min, after which it is cooled to room temperature and the colour read against a reagent blank at 540 mμ. A standard glucosamine solution, containing 100–300 μg/ml, is similarly treated.

RESULTS

(1) Examination of sound and carious dentine hydrolysates

In the autoclaved preparations 70 per cent of the carious dentine matrix went into solution. This solution was light brown, and there was no indication of the presence of black pigment particles which, if present, would have been released during the gelatinization of the major part of the matrix. The insoluble residue was no darker than the original carious dentine powder, which made it unlikely that the hypothetical pigment particles, if present, were confined to this residue. The equivalent autoclaved sound dentine preparations were colourless.

Similarly, the collagenase attack on carious dentine, which disintegrated 75 per cent of the matrix, was not associated with the release of any black precipitate. The sound dentine collagenase incubate was colourless, and the carious dentine incubate was light brown.

The acid hydrolysates of carious dentine, prepared with 5 N formic acid or 1·5 N sulphuric acid, were light brown, but unlike hydrolysates prepared with strong mineral acid (6 N HCl) they were devoid of any black precipitate. An alkaline hydrolysate, see (4) below, was also completely free of any black precipitate.

(2) Carbohydrate content of sound dentine and carious dentine

Different collections of pooled sound dentine and carious dentine were prepared and designated as SD1, SD2, CD1, CD2, etc. Determinations of carbohydrate content were made either on the demineralized powders or on partial hydrolysates using the methods described above. The carbohydrate content of each sample was then calculated on a percentage weight basis. As the nature of the carbohydrate material present was unknown, the carbohydrate content was arbitrarily expressed in "glucose" units for the α-naphthol, anthrone and cysteine methods of analysis. The concentration of "hexosamine" is arbitrarily expressed as "glucosamine" units.

The results of these analyses are summarized in Table 1.

Carbohydrate estimations were also carried out on the carious dentine fractions resistant to further solution by the collagenase preparation or the autoclaving procedure. These resistant residues were brought into solution by heating with 1·5 N

S_2HO_4, and the carbohydrate content estimated using the cysteine method. It was found that the collagenase resistant fraction contained 11·5% "glucose", and the fraction resistant to autoclaving contained 9·3% "glucose".

TABLE 1.

Dentine sample	Percentage Carbohydrate determinations*			Hexosamine content†
	α-Naphthol method	Anthrone method	Cysteine method	
CD1$_s$	1·2	4·2	3·8	—
CD2$_s$	2·5	5·1	4·9	—
CD3$_F$	1·7	3·7	3·6	—
CD4$_F$	1·8	3·8	3·2	—
CD5$_F$	1·4	4·1	3·8	—
CD6$_F$	2·2	4·2	4·0	—
CD7$_F$	1·5	3·2	2·8	—
Mean	1·8	4·0	3·7	
SD1$_s$	0·07	0·4	0·3	—
SD2$_F$	0·04	0·4	0·3	—
SD3$_F$	0·05	0·4	0·3	—
Mean	0·05	0·4	0·3	
CD1	—	—	—	2·6
CD2	—	—	—	1·7
CD4	—	—	—	1·7
CD5	—	—	—	2·2
Mean				2·0
SD1	—	—	—	0·3
SD2	—	—	—	0·3
Mean				0·3

* The carbohydrate contents are calculated on a percentage weight basis of "glucose" units present.

† The "hexosamine" contents are calculated on a percentage weight basis of "glucosamine" units present.

Hydrolysates prepared using formic acid are indicated by the subscript F (e.g. SD1$_F$) and hydrolysates with sulphuric acids by the subscript S (e.g. CD3$_S$).

(3) *Paper chromatography of carious dentine and sound dentine hydrolysates*

Since the results of the analyses indicated the presence in carious dentine of material giving a positive hexosamine (amino-sugar) reaction, attempts were made to separate and identify the possible hexosamine components, using paper chromatographic methods.

Samples of carious dentine hydrolysates containing, on the basis of the previously calculated content, sufficient "hexosamine" for subsequent detection were applied to strips of Whatman's No. 1 chromatography paper. These were then developed, using the solvent system l-butanol: acetic acid: water (4: 1: 5), and the dried chromatogram strips were then treated with either the acetylacetone Ehrlich reagent procedure of PARTRIDGE (1948) or with aniline hydrogen phthalate reagent (PARTRIDGE, 1949). With neither procedure was it possible to detect areas on the chromatograms corresponding in position to those of adjacent glucosamine and galactosamine markers run simultaneously. In a different experiment glucosamine or galactosamine was added to carious dentine and sound dentine powders in amounts corresponding to the calculated "hexosamine" content indicated by the Elson–Morgan reaction. Hydrolyses of 2 and 18 hr duration respectively were then carried out with N HCl, and subsequent chromatography of the dried acid-free products showed that both hexosamines could be detected on the paper chromatograms of the sound and carious dentine hydrolysates.

(4) *Direct Ehrlich reaction with alkaline hydrolysates of carious dentine*

Alkaline hydrolysates of demineralized carious dentine were prepared by heating 50 mg quantities with 5 ml of 2 N NaOH in sealed ampoules for 16 hr at 100°C. The hydrolysates were light brown, and no dark precipitate was present. Of the hydrolysates, 2 ml aliquots were removed and 2 ml of the Ehrlich reagent added. An immediate red colouration appeared which increased in intensity on standing. Absorption curves of the colour formed showed that the maximum absorption was at 560 mμ. A comparable alkaline hydrolysate of sound dentine was found to contain a small amount of material giving a direct Ehrlich reaction. It was present in quantities approximately one-fifth of that in the carious dentine hydrolysate.

Treatment of the 50 mg quantities of carious dentine with 5 ml 2 N NaOH or 5 ml 2 N Na$_2$CO$_3$ for periods of 30 or 60 min failed to give solutions giving a direct Ehrlich reaction. Carious dentine treated with 2 N NaOH for periods of 2 to 3 hr showed the presence of small amounts of material giving a positive Ehrlich reaction.

DISCUSSION

Hydrolysates of carious dentine prepared with strong mineral acids, such as 6 N HCl, have been found to contain significant amounts of a brownish-black precipitate (DEAKINS, 1941; ARMSTRONG, 1959). However, in the results reported above, it was found that autoclaved carious dentine solutions, and the enzymic, alkaline and mildly acidic hydrolysates of carious dentine did not show the presence of any dark precipitate. These observations indicate that the black particles present in acid hydrolysates

of carious dentine were probably formed during the hydrolysis, as a result of the strong acid medium employed.

As indicated in the introduction, this feature is characteristic of the acid hydrolysis of proteins containing significant quantities of carbohydrate. The possible presence of carbohydrate material in carious dentine was therefore tested for, using various analytical methods employed in the detection and estimation of carbohydrates.

The use of the reagents α-naphthol, anthrone and cysteine showed that carious dentine contained significant quantities of carbohydrate. The α-naphthol method indicated the presence of approximately 1·8 per cent of carbohydrate in the carious dentine matrix. The cysteine method gave values of about twice this value for carious dentine (mean: 3·7 per cent), while the anthrone method gave somewhat higher values (mean: 4·0 per cent). These differences doubtless reflect differences in the specificities of the methods used and, in conjunction with further investigations, may assist in indicating the nature of the carbohydrate material present in carious dentine.

However, without knowledge as to the nature of the components in carious dentine giving the positive carbohydrate reactions described, it is not possible at this stage to give an accurate estimate of the carbohydrate content in carious dentine other than to express it in arbitrary "glucose" units for each method used. The ratio of "glucose" values given by each carbohydrate method is not the same for each carious dentine preparation. This suggests that the components may vary qualitatively and/or quantitatively in different samples. If, as seems likely, the carbohydrate material is of bacterial, dietary or salivary origin, it might be expected that varying quantities of individual carbohydrate components would occur in different samples of carious dentine. It is considered probable that the α-naphthol method failed to detect some component or components which did give a positive reaction with the anthrone and cysteine methods. The assumption is made that the latter reactions give a more accurate measure of the total carbohydrate content.

Similar difficulties are encountered when examining the data for the Elson–Morgan reaction given by carious dentine. On the basis of the results obtained, a "hexosamine" content of 2 per cent was assigned to carious dentine, expressed in "glucosamine" units. However, it does not appear that the positive colouration given by carious dentine is due to the hexosamines likely to be present in biological material. Using paper chromatographic methods it was not possible to detect either glucosamine or galactosamine in carious dentine hydrolysates. Also, treatment of carious dentine with strong alkali gave an hydrolysate giving a direct Ehrlich reaction. The drastic alkaline treatment employed would destroy any free or acetylated hexosamine present in the matrix, and the fact that the material giving this positive Ehrlich reaction appeared only on prolonged alkaline treatment indicates that it is either slowly formed or released during the hydrolysis.

A further observation of interest was that the carious dentine fractions resistant to autoclaving or collagenase action contained considerably higher concentrations of carbohydrate. It may be that this association is fortuitous, but consideration must also be given to the possibility that the higher carbohydrate concentration might be related in some way to resistance to solution by autoclaving or collagenase attack.

The carbohydrate material in carious dentine may merely be present as a contaminant, arbitrarily adsorbed on to the matrix. Alternatively it may be attached at specific sites. It is known that collagen will intimately combine with certain mucopolysaccharides (BAZIN and DELAUNEY, 1957) and bacterial polysaccharides (BAZIN and DELAUNEY, 1958), and it has been shown that glucose can react with lysine amino groups to give compounds which, after treatment with alkali, give a positive Ehrlich reaction (GOTTSCHALK and PARTRIDGE, 1950). These latter compounds also readily form humins when treated with acids. It is also known that, under certain conditions, hydrolysis of proteinaceous material with a high carbohydrate content can produce compounds which give a positive Elson–Morgan reaction, and therefore interfere with hexosamine determinations (VASSEUR and IMMERS, 1952). These various factors are being considered in investigations which are being undertaken to determine the nature of the components responsible for the carbohydrate reactions given by carious dentine.

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AN ELECTROMAGNETIC BLOOD FLOWMETER FOR MEASURING BLOOD FLOW TO THE ORAL REGION OF ANAESTHETIZED EXPERIMENTAL ANIMALS

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Abstract—An A.C. electromagnetic blood flowmeter capable of recording the small volume flows occurring in the arteries supplying the oral tissues has been constructed. The cannula and the magnetic head construction require the interruption and cannulation of the vessels. The amplifier consists of five stages in a cascade arrangement and incorporates a small amount of negative feedback. The maximum amplifier gain is in excess of one million. The output, which is proportional to the volume blood flow, is recorded on an ink-writing oscillograph. The high amplifier gain and precise phase monitoring system permit blood-flow determinations as small as one millilitre per minute.

SINCE the work of KOLIN (1936) and WETTERER (1937) numerous modifications of the electromagnetic blood flowmeter have been developed. Monographs by GLASSER (1950) and by POTTER (1948) comprehensively survey the principles employed in construction of these systems. Most of these modifications have resulted in complex instruments which are difficult to duplicate and have been used principally to measure relatively large volume flows. The variable phase transformers of older designs (KOLIN, 1941) have largely been replaced by resistance–capacitance phase shift networks (COBBOLD and STYLES, 1955) or by phase discriminators in the absence of bucking voltages (DENISON, MERRILL and GREEN, 1955). Recently, a simplified electromagnetic blood flowmeter has been developed by RICHARDSON (1959) which employs a five stage cascade type amplifier and is suitable for recording large flows with an ink-writing oscillograph.

There is at present very little available information concerning the characteristics and importance of blood flow to the oral tissues. It has therefore been the purpose of this investigation to construct an electromagnetic blood flowmeter which is simple to operate and has sufficient stability and gain to measure small volume flows. The instrument would then provide a means continuously to monitor volume blood flows in the vessels supplying the oral cavity.

DESCRIPTION OF THE INSTRUMENT

Cannula. Cannulae were constructed from two polystyrene tubes. The smaller tube had an inside diameter of approximately 3·5 mm and was fitted with four platinum electrodes using 22 gauge wire (Fig. 1). The two widely separated electrodes

were connected to the electrical ground and the two centrally opposed electrodes were connected to the amplifier input by means of a flexible shielded cable. The larger polystyrene tube was fitted over the smaller one, after the electrodes were inserted and glued in place. Plastic brackets (Fig. 2) secured the cannula firmly in place within the jaws of the magnet.

FIG. 1. Construction of the cannula and pickup probes.

Magnet. The magnet was constructed of laminated iron arranged so that the air gap was 8 mm wide (Fig. 2). The excitation coil consisted of 2750 turns of No. 28 enamelled copper wire and was excited by 115 V a.c. The horizontal plates of the oscilloscope, which monitored phase, were driven by voltages developed from thirty turns of No. 28 wire placed around the upper arm of the magnet. Three turns of No. 28 wire placed around the lower arm of the magnet supplied the bucking voltages to the amplifier.

Amplifier. Voltages developed at the cannula pickup probes were led to the grid of the first amplifier tube to form a junction with the out-of-phase bucking voltage (Fig. 3). The use of an a.c. magnet for excitation of the cannula source introduces an input e.m.f. even when no flow is allowed through the cannula. In order to keep the deflection of the recorder at zero during periods of no flow, the bucking voltage must be equal and 180° out of phase with the no-flow cannula voltage. Proper cancellation is obtained by balancing a resistance–capacitance phase shift network. The amplified resultant of these two voltages is applied to the vertical plates of an oscilloscope whose horizontal sweep is activated by the voltage from a coil around the cannula magnet. Fig. 4(a) illustrates the sweep pattern with a properly phased circuit during no cannula flow. Fig. 4(b) illustrates the sweep pattern obtained with a properly phased circuit during passage of blood or saline through the cannula. The

SCHEMATIC OF AMPLIFIER
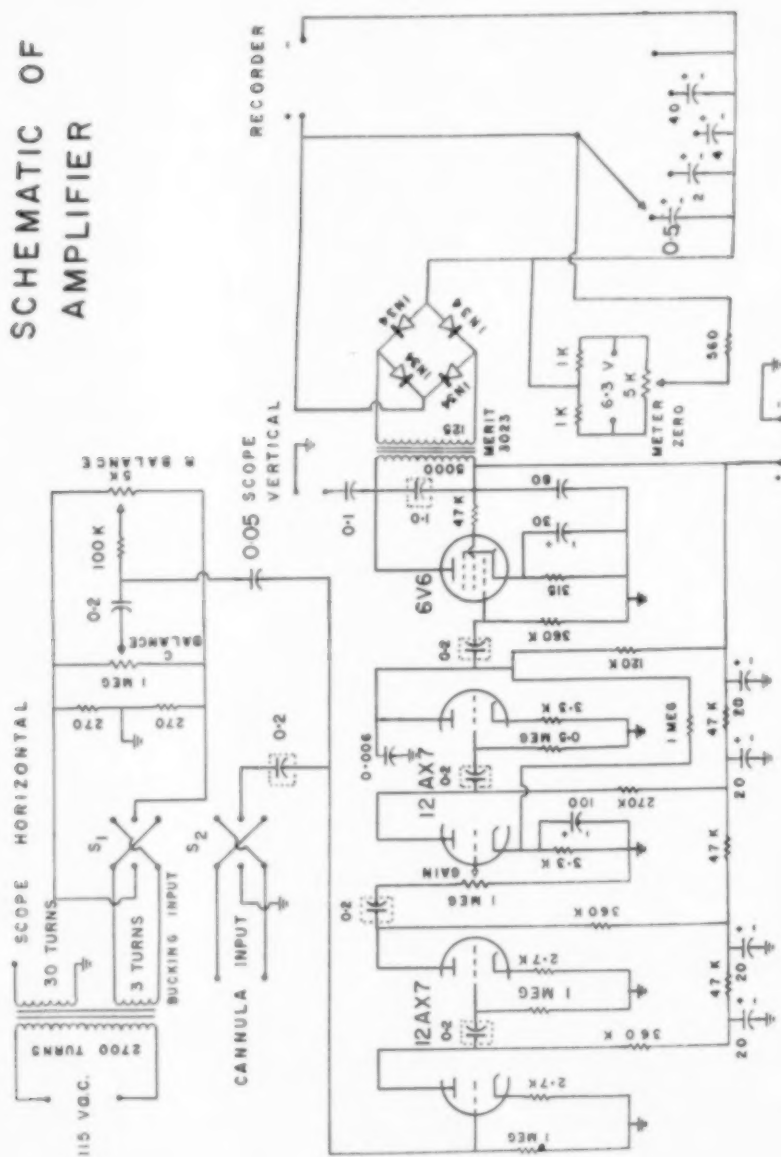
Fig. 3. Circuit diagram of the amplifier and cannula pickup probes. The amplifier must be supplied with well regulated d.c. power supplies, both for the B+ and the filament voltages.

vertical deflection is proportional to the amount of the volume flow and the amplifier gain.

Following the initial phase adjustment, the cannula input voltage exceeds the bucking voltage only when flow is allowed in the cannula. The voltage difference is amplified by five amplifier stages in a cascade arrangement. The amplifier incorporates a small amount of negative feed back between stages 3 and 5. The maximum amplifier gain is in excess of one million and is controlled by the potentiometer between stages 2 and 3.

Recorder. The output voltage from the amplifier is led to a full-wave rectifier circuit through an output transformer and then passed to an Esterline Angus Recorder. A zero balance network and damping capacitors are inserted between the rectifiers and the recorder. The 5 mA recorder has a d.c. resistance of approximately 80 Ω, therefore 0·4 V output from the amplifier are required to produce a full-scale deflection of the recorder.

ELECTRICAL CHARACTERISTICS OF THE INSTRUMENT

Determination of the voltages developed in the cannula probes was accomplished by applying small known voltages to the amplifier in place of the usual cannula. A constant gain setting was maintained and the deflection of the milliampere meter was recorded. Graphic analysis (Fig. 5) of the input voltage on the ordinate and the milliamperes deflection on the abscissa provided a comparison between the known voltages and the voltages developed by known flow rates. These data show that approximately 0·11 μV are produced in the cannula for each millilitre per minute flow of normal saline, but that substitution of blood for the saline increases the cannula voltage to approximately 0·43 μV for each millilitre per minute. Therefore, recording of blood flows in the range of one millilitre per minute requires that the total amplifier noise level be lower than 0·43 μV at the input stage.
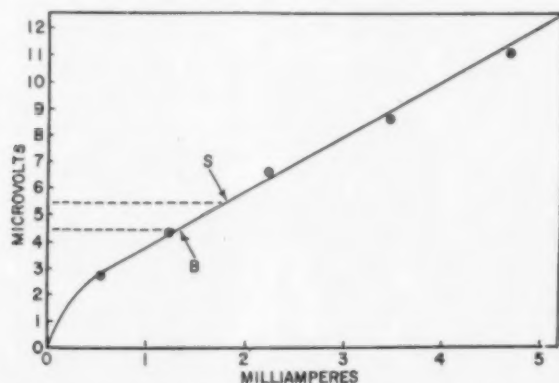
Fig. 5. Relationship of the milliamperes deflection of the recorder obtained with various known input voltages and with known flow rates. S—Deflection of recorder produced by 88 ml/min normal saline. B—Deflection of recorder produced by 15 ml/min blood.

Application of increasing increments of known voltages (Fig. 5) demonstrates that the amplifier response is linear in all ranges above 0·5 mA. The linear part of the range is independent of amplifier gain; therefore, the input voltage, at which the small non-linearity appears, decreases with increasing gain settings. This characteristic has been traced to the IN34 diodes used in the rectifier circuit. The instrument demonstrates stability at all amplifier gains. Drift of the zero baseline does not occur to a measurable extent when saline is used or when standard voltages replaced the cannula input, but can occur when working with blood at very low volume flow rates. This feature necessitates periodic determination of the baseline during its physiological use. Baselines are determined by briefly clamping the tubing supplying the blood to the cannula.

Rectification and capacitive smoothing in the output circuit are required, since the signal developed at the cannula source is a.c. These components introduce resistive–capacitive time constants which can be kept to low values and hence do not interfere with the recording of moderately rapid changes in mean flow rates. These time constants also serve to integrate the cyclic flow changes into mean flow. Rapid changes which occur during each heart cycle, particularly in large elastic arteries, have durations shorter than the meter time constants and hence are damped out in the flow recording. These characteristics were cited by INOUYE and KUGA (1954) for other electromagnetic blood flowmeters.

APPLICATION OF THE INSTRUMENT

Measurements of small volume flows with electromagnetic blood flowmeters have been previously neglected, principally because of cannulation difficulties and instrumentation problems involving excessive zero drift and instability at high gains. The meter described has been successfully employed in the determination of mean flow in the mandibular and maxillary arteries of dogs (BISHOP, MATTHEWS, DORMAN and
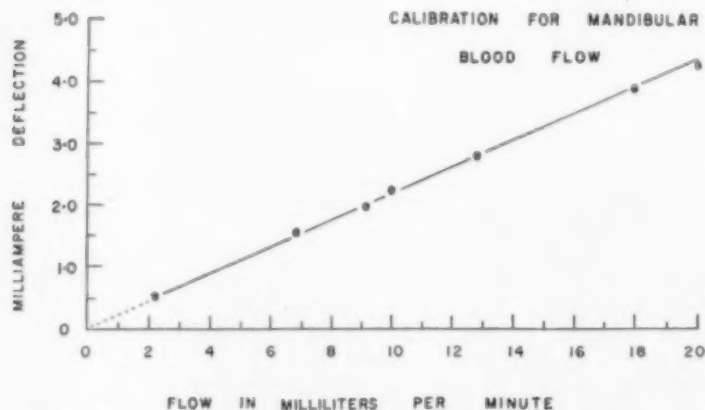
FIG. 6. Calibration curve produced by collecting blood from the opened distal side of the cannula into graduated vessels for 15 sec intervals.

MOORE, 1959; MATTHEWS, BISHOP, MOORE and DORMAN, 1959). Volume flow rates as low as one millilitre per minute were recorded. A calibration curve similar to that in Fig. 6 was made following each experimental determination of flow. This calibration technique required detachment of the distal side of the polyethylene cannula and collection of blood for 15 sec intervals in graduated vessels. Fig. 7 shows a typical record of blood flow in a small artery. Two zero points were obtained by occluding the proximal side of the polyethylene tube which supplied blood to the cannula. No zero drift is apparent. Each flow interruption is followed by a transient rise in volume flow which corresponds to reactive hyperemia (RANDALL and HORWATH, 1953). Individual peaks are seen for each heart beat and are superposed upon the slower respiratory waves. Mean flow values at any point on the record are determined by selecting the value midway between the maximum systolic and minimum diastolic excursions of the recording needle. Use of the meter in combination with pressure recording allows calculation of peripheral resistance (GREEN, LEWIS, NICKERSON and HELLER, 1944).
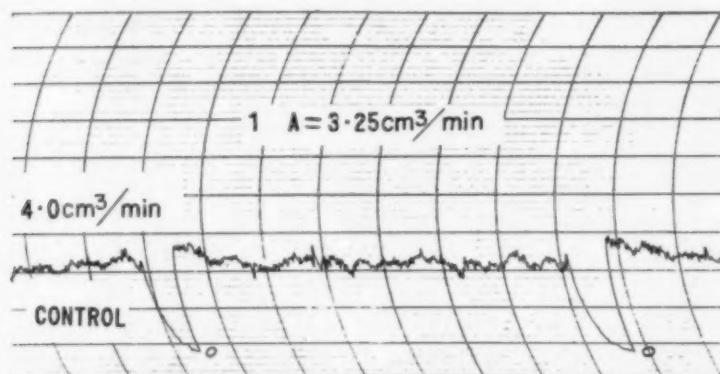
FIG. 7. Typical record obtained with this instrument from the mandibular artery of dogs.

The range of blood-vessel lumen diameters exhibits a continuous spectrum beginning with the vena cava and aorta and ending in the smallest capillaries. Peripheral resistance of the vascular components increases rapidly as the lumen diameter decreases. Therefore, interpretations of flow and pressure measurements made in large vessels in terms of peripheral resistance units are limited to mean values of great variety of varying resistances. Development of an instrument which permits determination of the peripheral resistance in more localized areas may be expected to supply information which will result in a better understanding of local tissue haemodynamics.

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FIG. 2.   Arrangement of the cannula in the magnetic field.

FIG. 4a.   Oscilloscope pattern produced by proper phase settings during no-flow conditions in the cannula.

FIG. 4b.   Oscilloscope pattern produced by flow in the cannula with proper phase settings. Pulsatile flow causes the open loop to oscillate in phase with the irregular flow surges.

PLATE 1
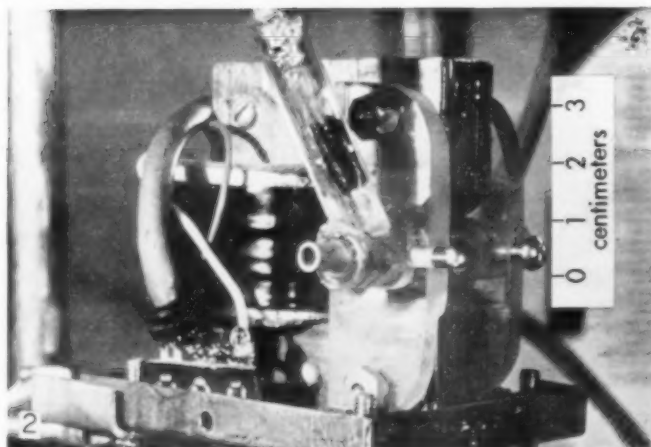
SHORT COMMUNICATION

DENTAL CALCULUS AND ANTIBIOTICS IN THE WHITE RAT

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DURING the course of a study on the oral lactobacilli of the rat (JORDAN, FITZGERALD and FABER, 1959), extensive calcareous deposits were observed on the teeth of animals receiving noncariogenic diet 550 of ZIPKIN and McCLURE (1952). The percentage composition of this diet is cornstarch 66·5, whole milk powder 27·0, whole dried yeast 5·0, cod-liver oil 1·0 and salt mixture 0·5. The salt mixture consisted of sodium chloride 500 gm, ferric citrate trihydrate 53·2 gm and copper sulphate pentahydrate 3·9 gm.

In the present study three groups of twenty-four Sprague–Dawley female rats received distilled water and diet 550 *ad libitum* from weaning. Chloramphenicol was mixed in the diet of one group at a level of 50 mg/kg. The second group received the same level of chlortetracycline, and the final group served as controls. A parallel series of animals, similarly treated, received the cariogenic coarse particle diet 585 of STEPHAN *et al.* (1952). After 110 days all surviving animals were sacrificed. The heads were autoclaved and the soft tissues were removed.

In every animal in each of the groups receiving diet 550 hard calcareous deposits were noted on the molar teeth. These ranged from discrete concretions on the mesial surfaces of the first maxillary molars (Fig. 1) to heavy deposits which completely invested the crowns of all of the maxillary and mandibular molars (Fig. 2).

Using an arbitrary scoring system of 0 to 5 for severity of deposits, the chlortetra-cycline group averaged 3·9 and the chloramphenicol and control groups each averaged 3·5. Corresponding groups of animals receiving cariogenic diet 585 had a score of 1·0, 0·9 and 1·2 for the chlortetracycline, chloramphenicol and control groups respectively.

Analysis of the pooled dental deposits yielded the following percentage values:

Animals on diet 550: ash, 58·6; Ca, 22·4; P, 9·7; Mg, 0·5;

CO_2, 10·3; N, 2·4

Animals on diet 585: ash, 51·3; Ca, 20·4; P, 8·2; Mg, 0·7; CO_2, 7·5.

The dietary calcium and phosphorus content was comparable for the two diets. On a percentage basis they were: Diet 550, Ca, 0·25; P, 0·28; Diet 585, Ca, 0·29; P, 0·27.

Samples of the crude and ashed deposits were subjected to electron and X-ray diffraction.* The resulting diffraction patterns coincided with those for hydroxy-apatite. Thus these calcareous deposits in the rat may be considered analogous to the dental calculus of humans.

It is noteworthy that neither chloramphenicol nor chlortetracycline, antibiotics capable of inhibiting a broad spectrum of micro-organisms, exerted any inhibitory effects on the deposition of calculus in the animals receiving diet 550. The same levels of these drugs in cariogenic diet 585, while not appreciably altering the slight degree of calculus deposition, markedly inhibited the development of caries. The caries scores were 3·7, 10·6 and 21·7 for the chlortetracycline, chloramphenicol and control groups respectively.

Gram-stained smears of the residue from the decalcified calculus revealed the presence of much amorphous material, cellular debris and micro-organisms of various types. In the smears from the antibiotic groups there appeared to be a preponderance of gram-negative rods with lesser numbers of gram-positive cocci and pleomorphic rods, whereas in the control groups the gram-positive elements predominated.

The significance of these results in relation to the possible role of the oral micro-flora in calculus deposition remains to be established. If specific organisms are involved in this process they are apparently unaffected by dietary levels of chlortetra-cycline and chloramphenicol high enough to inhibit the cariogenic microflora.

* The X-ray diffraction patterns were obtained through the courtesy of V. M. MOSELY using copper radiation.

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ZIPKIN, I. and MCCLURE, F. J. 1952. Deposition of fluorine in the bones and teeth of the growing rat. *J. Nutr.* **47**, 611–619.

FIG. 1. Calcareous deposit on first maxillary molar of the rat. According to the arbitrary scoring system used this would be assigned a numerical value of one.

FIG. 2. Calcareous deposit covering entire crown of first maxillary molar. The same heavy deposit covered the second and third molars as well and would be assigned a score of five.

PLATE 1

CURRENT PAPERS IN ORAL BIOLOGY

Honorary Editor: Maurice V. Stack

The first number of the *Archives* included notes on the classification of publications to be listed, and the next three numbers, completing Volume 1, each contained two hundred titles. It is hoped that the scheme of classification and the scale of selection from the accessible literature are providing the specialist with the means to trace recent research publications in oral biology.

The present *Current Papers Section* offers a re-statement of the types of research included under each heading, together with a list of abbreviated titles of those journals from which more than a single title has been selected; the fourth *Section* is to appear in the next number of the *Archives*. Figures in parentheses following notes on the twelve headings show the totals of publications making up the eight hundred so far classified:—

(1) **Anatomy.** Structures and relationships of hard and soft tissues. (84)

(2) **Histology, Normal.** Studies using electron microscopy, histochemistry, microradiography, autoradiography, etc. (73)

(3) **Physiology.** Functions of oral tissues and salivary glands. (55)

(4) **Biochemistry.** Includes solubility studies, crystal structure, etc. (83)

(5) **Genetics.** Inherited characteristics and anomalies of structures. (22)

(6) **Histopathology.** Studies involving techniques as in (2) above. (53)

(7) **Experimental Pathology.** Induced structural and functional changes. (113)

(8) **Caries.** Publications bearing directly on dental decay processes. (101)

(9) **Microbiology.** Studies of oral microflora and relevant antibiotics. (50)

(10) **Epidemiology and Clinical Studies.** Includes surveys of distribution, incidence and treatment of oral disorders affecting groups of cases. (92)

(11) **Materials and Technique.** Listing advances in research methods. (54)

(12) **Miscellaneous.** Contributions not directly classifiable under the above. (20)

Abbreviated titles of journals published throughout the world from which more than one title is quoted in the first four sections (800 titles).

Acta morph., Acad. Sci. hung.
Acta odont. scand.
Acta paediat., Uppsala
Actualités odonto-stomat.
Amer. J. Anat.
Amer. J. Orthodont.
Amer. J. Physiol.
Amer. J. publ. Hlth
Anat. Rec.
Angle Orthodont.
Ann. R. Coll. Surg. Engl.
Ann. Soc. belge Méd. trop.
Arch. Biochem. Biophys.
Aust. dent. J.
Biochem. J.
Biochim. biophys. Acta
Biol. Neonat.
Brit. dent. J.
Bull. Gr. int. Rech. Stomat.
Bull. Northw. Univ. dent. Sch.

Cah. odonto-stomat.
Čsl. Stomat.
Czas. stomat.
Dent. Practit. Rec.
Dtsch. Z. ges. gerichtl. Med.
Dtsch. Zahn-, Mund- u. Kieferheilk.
Dtsch. zahnärztl. Z.
Fogorv. Szle
Gig. i Sanit.
Helv. odont. Acta
Hum. Biol.
Int. dent. J.
J. Amer. dent. Ass.
J. Anat., Lond.
J. Bact.
J. Bone Jt Surg.
J. Canad. dent. Ass.
J. dent. Med.
J. dent. Res.
J. Jap. stomat. Soc.

J. nerv. ment. Dis.
J. Nutr.
J. Okayama med. Ass.
J. oral Surg.
J. Path. Bact.
J. Periodont.
J. Pharmacol.
J. prosth. Dent.
J.R. micr. Soc.
J. Ultrastruct. Res.
Kokubyo Z.
Mikroskopie
Nature, Lond.
N.Z. dent. J.
N.Y. St. dent. J.
Odont. Revy
Odont. Tidskr.
Oral Surg.
Plast. reconstr. Surg.
Proc. Nutr. Soc.

Proc. R. Soc. Med.
Proc. Soc. exp. Biol., N.Y.
Rev. belge Stomat.
Rev. brasil. Odontol.
Rev. franç. Odonto-Stomat.
Schweiz. med. Wschr.
Schweiz. Mschr. Zahnheilk.
Science
Sech. physiol. J., USSR
S. Afr. med. J.
Stain Tech.
Stoma
Stomatol., Liège
Stomatologiia, Moskva
Surg. Gynec. Obstet.
Svensk. Tandläk. Tidskr.
Tandlaegebladet
Tidskr. Tandheelk.
Vjschr. naturf. Ges. Zürich
Zahnärztl. Praxis

ERRATA

H. F. BAUMGÄRTNER und H. D. CREMER: Verschiedene Methoden zur Bestimmung der Phosphatase-aktivität in Rinderzahnen

In this paper (*Arch. oral Biol.* **1**, 8–22) Dr Baumgärtner's initials should have been given as H. F., and on p. 14, line 4, the word "linear" should have appeared after ". . . bis zu 30 Minuten".

SOME OBSERVATIONS ON UNGEWITTER'S METHOD FOR STAINING NERVE FIBRES AND NERVE ENDINGS IN TISSUE SECTIONS

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Abstract—The mechanism of the staining of nerve fibres in diethylene glycol distearate embedded sections of formalin-fixed decalcified teeth by the method of UNGEWITTER has been investigated. The variable results which have been obtained with this method were shown to be due to the changes in chemical composition of the solutions resulting from the instability of urea in aqueous solution, and could be explained in terms of current theories of nerve staining.

INTRODUCTION

IN 1951 UNGEWITTER introduced a silver staining method for the demonstration of nerve fibres and nerve endings in paraffin sections of soft tissues. In this method a novel constituent, urea, was introduced into the impregnating and developing solutions. Although UNGEWITTER arrived at the method by empirical trial, its advantages were believed to be due to the effect of some physico-chemical properties of urea in high concentration (20–30%) in aqueous solution. These effects were stated to be (a) alteration of the physical state of the tissue itself, (b) acceleration of the staining reaction, (c) suppression of the ionization of the silver nitrate, and (d) retardation of the formation of large aggregates of silver thus giving fine grain. No direct experimental evidence was offered for the occurrence of such effects in the staining solutions. It was found essential to include small amounts of picric acid and mercuric cyanide in the impregnating solution and, in order to achieve the required intensity of staining, the process of impregnation and development was repeated, if necessary, several times.

POWERS (1952) applied the method to decalcified sections of teeth, and found it the only one out of six methods used which produced specific staining of nerve fibres. COCKER and HATTON (1955) have also used this method for staining paraffin sections of decalcified teeth, but found that the staining was improved if a few drops of pyridine were added to the impregnating solution. Over a period of two years UNGEWITTER's technique was used in the Department of Dental Pathology, University of Birmingham, for staining decalcified serial sections of teeth embedded in diethylene glycol distearate (BRAIN, 1955). While excellent examples of nerve staining have been obtained, the results were inconsistent.

This variation appeared to be due to changes in the properties of the staining solutions. Using a single impregnation and development, little or no staining was obtained with freshly prepared solutions. The staining was increased if both the

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developing solution and the 40% stock urea solution, from which the impregnating solution was prepared, had stood for several weeks at room temperature. These "mature" impregnating solutions were characterized by the formation during mixing of a voluminous white precipitate (later shown to be silver cyanate). The "maturation" could be achieved more easily by heating the stock urea solution in a boiling water bath, or by previous incubation of the impregnating solution for several hours. The use of a freshly prepared impregnating solution with a mature developer, or alternatively a mature impregnating solution with a freshly prepared developer, also produced little or no staining.

The technique of repeated impregnation and development could not be relied on to improve the nerve staining, and frequently it produced merely an increase in general staining. In view of the excellent results which could, on occasion, be obtained, an investigation of the chemical properties of the solutions was carried out in an attempt to correlate changes of the staining properties with changes in chemical composition.

The chemical basis for silver staining of nerves

The work of HOLMES (1943), SAMUEL (1953a,b) and PETERS (1955a,b,c,d) has considerably increased our understanding of the mechanism of the silver staining of nerves. These workers conclude that discrete staining of tissue components depends upon the formation of nuclei of reduced silver in these components during impregnation. The bulk of the silver (reducible silver) taken up by the section is loosely and reversibly bound, probably by the amino acid histidine, and is generally distributed. The final visible stain is produced by development in a reducing solution of suitable composition, during which process reducible silver is deposited in the reduced form on the silver nuclei present.

At constant temperature the number and distribution of the silver nuclei formed, which govern the specificity and spectrum of the final stain, depend upon and increase with the time of impregnation and with the silver ion and hydrogen ion concentrations of the impregnating solution. The amount of reducible silver taken up also increases with the silver ion and hydrogen ion concentrations of the impregnating solution but is practically complete after 10 min at 37°C (PETERS, 1955a).

The development process is controlled by a number of factors including the chemical nature of the developing agent, and the sulphite and hydrogen ion concentrations of the solution.

Considering the technique of repeated impregnation and development introduced by Ungewitter in terms of this mechanism, it is evident that the silver deposits produced during the first development act as silver nuclei during subsequent development and are likely to outweigh the effect of any new silver nuclei formed during further impregnation. The effect of this process, therefore, is to intensify existing staining. If specific staining of the nerves is present after the first impregnation then the contrast will be emphasized by further treatment, but if the staining is general initially then no improvement of the nerve staining can be expected by repetition of the staining process.

Chemical properties of Ungewitter solutions

The chemical investigation was confined to a study of cyanate and hydrogen ion concentrations of urea solutions, silver, silver ion and hydrogen ion concentrations of Ungewitter impregnating solutions, and hydrogen ion concentrations of Ungewitter developing solutions. Total silver was determined by Volhard titration. Silver and hydrogen ion concentrations were determined electrometrically, using silver and glass electrodes respectively. Cyanate was determined by argentimetric titration.

(a) *Urea solutions.* In aqueous solutions urea undergoes a tautomeric change with the formation of ammonium cyanate. In 40% w/v urea (6·7 M) an equilibrium concentration of 0·7% ammonium cyanate was reached after 15 min at 100°C. At lower temperatures the rate of transformation is very slow (DIRNHÜBER and SCHÜTZ, 1948). At room temperature concentrations of 0·08–0·13% cyanate had formed in stock 40% solutions of urea which had been stored for 6–36 weeks. Some hydrolysis of the cyanate to ammonium carbonate occurred in these solutions, causing the pH to rise to 8·3–9·0. No detectable changes occurred when the solutions were stored at 0°C., but at the impregnation temperature, 60°C., the formation of cyanate is fairly rapid.

(b) *Impregnating solutions.* The chemical composition of the impregnating solutions and the changes of composition occurring during incubation at 60°C. are illustrated in Fig. 1, in which the concentrations are expressed in logarithmic form.
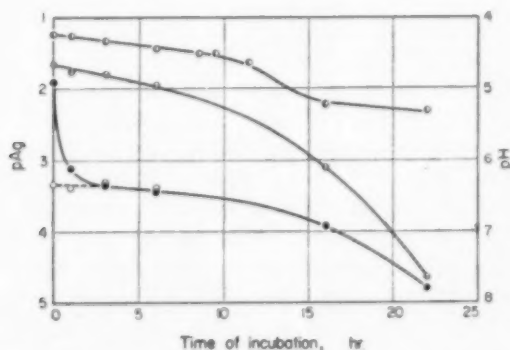
Fig. 1. The silver (◐), silver ion (⊖) and hydrogen ion (fresh solutions ●, mature solutions ○) concentrations of an Ungewitter impregnating solution incubated at 60°C. The initial solution was prepared from 20 ml 2% silver nitrate (AnalaR), 20 ml 40% urea (AnalaR) and 0·17 ml 1% picric acid/1% mercuric cyanide.

The initial pH of the impregnating solutions (Table 1) showed considerable variation according to the sample of urea in the preparation and the amount of picric acid–mercuric cyanide solution added. Fresh solutions showed a variable and relatively acid reaction due partly to a slight reaction between the urea and silver ions which liberates hydrogen ions and partly to the picric acid, coupled with negligible buffering power of the solution. Small amounts of basic impurities in the urea samples can

materially affect the initial pH of the solution. Upon incubation the pH of fresh solutions rises rapidly. This is due to the hydrolysis to ammonia and carbon dioxide of cyanate, formed from urea, which is unstable in the acid solution. As the pH increases the cyanate becomes more stable (DIRNHÜBER and SCHÜTZ, 1948) and the pH rises more slowly to that of the mature solutions, that is to about 6·4 and increases only slowly upon incubation. The higher pH of the mature solutions is due to the buffering action of the ammonium carbonate developed in the stock urea solutions.

TABLE 1. THE INITIAL pH VALUES OF UNGEWITTER IMPREGNATING SOLUTIONS.
THE SOLUTIONS CONTAINED 1 % SILVER NITRATE, 20 % UREA, AND 3 DROPS
1 % PICRIC ACID—1 % MERCURIC CYANIDE SOLUTION PER 100 ml

Urea Sample	pH	
	Without picric acid	With picric acid
AnalaR, recrystallized twice from alcohol	5·0	4·7
AnalaR, fresh sample	5·4	4·9
AnalaR, stored in laboratory 2 years	5·9	5·2
Mature solution (40%)	6·4	6·4

The interaction between silver nitrate and the urea also reduces the silver ion concentration to about half that of the total silver. There is little difference between silver and silver ion concentrations of fresh and mature solutions. With the formation of cyanate upon incubation the sparingly soluble silver cyanate is precipitated and both silver and silver ion concentrations decrease steadily. The removal of the cyanate from solution as it is formed minimizes its hydrolysis to ammonia and carbon dioxide. This gives rise to a region of comparative pH stability. When, after many hours incubation, the excess silver has been precipitated, the free cyanate concentration increases and hydrolysis causes the pH to rise more rapidly. The silver ion concentration decreases increasingly rapidly but the total silver concentration falls to a minimum (and eventually increases) owing to the formation of soluble complexes.

(c) *Developing solutions*. The pH of a developing solution containing 2% hydroquinone, 10% anhydrous sodium sulphite and 20% urea, when stored at room temperature in dark glass bottles, rose from 8·7 when freshly prepared, to 9·1 after 5 weeks and 9·3 after 9 weeks. The change appears to be due to the formation of ammonium carbonate from the urea and the smell of ammonia was strongly evident in the mature developer. The increase of pH was accompanied by the production of staining properties when used in conjunction with impregnation by mature solutions. Similar staining properties could be obtained by adjusting the pH of fresh developing solutions with ammonia to 9·2–9·4.

DISCUSSION

The difference between the conditions of impregnation in fresh and mature solutions is principally one of pH. At pH 4·5 and 5·5 PETERS (1955a) obtained only non-specific staining and considered that nuclei formed in these acid solutions did not give rise to differential staining. Thus discrete staining of the sections is unlikely to result if the initial impregnating solution is too acid. Since repeated impregnation and development only intensifies the existing staining this treatment only increases the non-specific staining.

In sections impregnated for 1 hr in mature solutions, or in fresh solutions the pH of which has been adjusted beforehand, fairly intense differential staining is obtained. This is not entirely specific for nerves since some other cell structures are also discretely stained. Since the nerve fibres appear to be the tissue elements which form silver nuclei most readily (below pH 8) these observations suggest that the optimal pH for nerve staining in the Ungewitter impregnating solution is in the region 5·5–6·0. Such a pH level may be attained, fortuitously, during the initial impregnation of fresh solutions to which little picric acid has been added, or which have been prepared with slightly impure urea. The addition of a small amount of the weak base, pyridine, to the fresh impregnating solution (COCKER and HATTON, 1955) probably has a similar effect.

Modifications of the impregnating solution

Although the mature impregnating solution did not give entirely specific nerve staining its staining properties are fairly consistent because of the relatively constant pH and silver ion concentration. It was noted that the background staining, which is probably due to the reduction *in situ* of some reducible silver, was diminished considerably when the impregnating solution had been preincubated for about 16 hr, and the nerve staining, although not as intense, was specific. This result appeared to be due to the reduction in the silver ion concentration, the solutions having a composition approximating to saturated solutions of silver cyanate in urea. Solutions having a similar composition prepared directly (0·1 % silver nitrate; 20 % urea (mature solution); 25 % 0·1 M maleate buffer pH 5·7–6·2) gave satisfactory nerve staining after two impregnations. The inclusion of buffer is necessary since, in the absence of an excess of silver, the pH changes fairly rapidly upon incubation. In these solutions picric acid–mercuric cyanide solution had no effect upon the staining and was therefore omitted.

Effects on staining of the presence of silver cyanate

Mature impregnating solutions, in addition to showing a higher pH than fresh solutions, differ in containing silver cyanate. Some observations suggest that the presence of silver cyanate has an effect on the silver staining of the section. Thus the staining given by a mature solution was more intense than that given by a fresh solution adjusted to the same initial pH. A similar effect was observed with the modified impregnating solution (pH 6·1). Since it could not be proved that the conditions of impregnation at 60°C were identical with respect to silver ion and

hydrogen ion concentrations, impregnations were carried out at room temperature. A modified solution (− cyanate) made up with fresh urea (recrystallized twice from alcohol) was brought to the same hydrogen and silver ion concentrations as a solution made up with mature urea (+ cyanate).

The nerve staining obtained with pairs of slides bearing sections cut from the same tooth is shown in Table 2.

TABLE 2.   THE EFFECT OF THE PRESENCE OF SILVER CYANATE ON THE NERVE
STAINING BY MODIFIED UNGEWITTER IMPREGNATING SOLUTIONS AT
ROOM TEMPERATURE

Impregnation period	Nerve staining	
	−Cyanate	+Cyanate
16 hr	Nil	+
40 hr	Nil	+ +
16 hr followed by second impregnation of 3 hr	+	+ +

The ionic concentrations in the solutions were similar after the impregnation. The silver cyanate appears to have a catalytic effect upon the staining although no mechanism for this can be suggested.

Functions of urea in the staining solutions

Of the possible functions of urea in promoting the silver staining the present observations provide evidence for only one—suppression of the ionization of the silver nitrate. When the staining of the mature Ungewitter solution was compared with that given by 1% silver nitrate at pH 6·4, the latter solution produced only intense general staining. However, lower concentrations of silver nitrate produced differential staining in the absence of urea (as has been shown by SAMUEL, 1953a, and others). Urea appears to play only a secondary part in the production of conditions suitable for nerve staining and the essential factor—the balance between hydrogen ion and silver ion concentration—is controlled by the formation and hydrolysis of cyanate derived from the urea.

Again, in the developing solution it is the decomposition products of the urea which produce the correct conditions for development and not the urea itself.

It was concluded, therefore, that the presence of urea in the staining solutions had no particular merit, and that suitable conditions for nerve impregnation might well be produced in its absence. Since its lability in aqueous solution is the cause of the instability of the solutions, its omission would allow stabilization of the solutions. It was found that saturated solutions of silver cyanate in dilute buffer, or even in water alone, gave at least as good nerve staining as similar solutions containing urea under comparable conditions. A further development of this modification, combined with the Ungewitter technique of repeated applications of

impregnation and development, has produced a method which has given excellent and consistent nerve staining with sections from a variety of teeth (ROWLES and BRAIN, 1960).

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THE EFFECT OF DENTINE FRAGMENTS ON THE HEALING OF THE EXPOSED PULP

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Abstract—The effect of dentine chips and fragments on the healing of the exposed pulp was studied in forty-six primary and permanent teeth. Findings of inflammation of various degrees, resorption of dentine chips and fragments and their encapsulation in "callus" showed a foreign body reaction by the pulp. In both inflamed and non-inflamed pulps the presence of dentine chips and fragments disturbed the healing and therefore the formation of dentine bridges was defective or absent.

VARIOUS reports of experiments with human and with animal teeth have indicated that dentine fragments which had been accidentally forced into the pulp became embedded in "callus" (KRONFELD, 1929) or "osteoid tissue" (FELDMANN, 1931). Moreover, it has been stated that the presence of these chips favoured the formation of secondary dentine (DÄTWYLER, 1921) and rapid calcification at the amputated surface (HELLNER, 1930). This observation has led some investigators to elaborate on methods for the use of dentine chips as a dressing material in cases of pulpotomy and pulp capping.

The results of pulp treatment reported for this method were variable. NEUWIRT (1928, 1933) and PRIBYL (1931), who examined treated pulps not only clinically but also histologically, reported all cases as successful. On the other hand, ZAJFE and SCHATZKER (1938) stated that seventeen out of eighteen cases were failures, since rarefaction of periapical bone was demonstrated radiographically. Also LOEWENSTEIN (1934) observed thirty-one failures from forty-eight cases. FELDMANN (1932), HELLNER (1930, 1933) and HOFFMANN (1937), who have advocated the use of dentine chips, reported from histological studies the presence of suppuration in many cases, even though "callus" and new dentine had formed.

Failures with a "dentine chip" method have sometimes been considered to be due to an "inadequate" operative technique (CASTAGNOLA, 1953; HELLNER, 1930; WILLNER, 1935). (Castagnola's book contains many references to the treatment of the exposed pulp with dentine fragments or calcium hydroxide.) In spite of the use of the rubber dam, careful removal of all softened carious dentine and disinfection of the cavity walls, KALNINS' and JAKOVLEVA'S (1938–39) unpublished findings on capping with dentine chips showed unfavourable results similar to those reported by ZAJFE and SCHATZKER (1938). In KALNINS' and JAKOVLEVA'S

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experiment, twenty-six pulps in teeth of adults and children were exposed because of deep caries. The treatment was performed according to the method advocated by NEUWIRT (1928, 1933) and PRIBYL (1931). After dressing with dentine chips, the pulps were sealed off with sterile gold foil covered with zinc phosphate cement or zinc oxide–eugenol. Pulps of only two treated teeth remained vital and asymptomatic during the period of one year. All others post-operatively developed pulpitis or necrosis with accompanying periodontitis within several weeks or months.

In a further experiment where pulps were also exposed because of deep caries or serous pulpitis but where a calcium hydroxide–sulphathiazole preparation (with addition of X-ray contrast medium-strontium salts) was employed for capping, KALNINS (1955) obtained results in which 85 per cent were clinically successful out of 182 treated cases. In all these cases care was used to remove dentine particles within all possible limits. Also, CASTAGNOLA (1953), GLASS and ZANDER (1949) and VAN HUYSEN and BOYD (1953), who used dressing materials other than dentine fragments, reported contradictory results on the favourable or disturbing effect of accidentally inserted dentine fragments on the healing of the exposed pulp.

This paper deals with the histological appearance of alterations occurring in pulp healing associated with dentine chips and fragments accidentally inserted into the pulp wound during the application of a calcium hydroxide paste.

MATERIAL AND METHODS

The histological material was provided from experiments by KALNINS (1955, 1957) on the treatment of the exposed pulp in primary and permanent human teeth, which were to be extracted for orthodontic or prosthetic reasons. The teeth were clinically intact or showed only small carious lesions. For the most part the cavity preparations involved the occlusal surface only. Since earlier experiments of KALNINS and JAKOVLEVA (1938–39), ZAJFE and SCHATZKER (1938) and LOEWENSTEIN (1934) (where dentine fragments were used in pulp capping) were unsuccessful, great care was employed to wash out with a water spray all dentine particles so far as possible from the cavity. After application of a 10 % iodine solution upon the pulpal roof, the pulp was exposed. The opening in the roof of the pulp chamber was enlarged sufficiently to permit a more complete elimination of dentine chips. Copious pulpal bleeding afforded a further cleansing action. Finally the pulp wound was cleaned and bleeding was stopped by the use of 3 % hydrogen peroxide solution. A capping paste of calcium hydroxide and sulphathiazole was then applied. In order to prevent the development of post-operative haemorrhage under the dressing the paste was pressed on the pulp with a cotton pellet.

Even with these precautions, sections showed that in thirty-nine out of seventy-one teeth (with post-operative period from 1 day to 14 months) dentine fragments were present at the site of the pulp wound. These thirty-nine cases offer the principal material presented in this paper. In addition there are included seven permanent and primary teeth from other experiments in which the pulp had been capped with calcium hydroxide.

FINDINGS

Table 1 shows that out of forty-six pulps with dentine fragments present, twenty-nine showed inflammation. In eighteen cases a diffuse pulpitis (with prevalence of neutrophils) or abscess was apparently caused by insertion of numerous small chips at the wound site (Fig. 1), or by their deeper dissemination into the pulp. Large dentine fragments tended to cause an inflammatory reaction characterized by an accumulation of lymphocytes, plasma cells and macrophages (Figs. 4, 5, 6, 11). The inflammation was regularly localized in the more coronal portion of the pulp, with the exception of three primary teeth. In these teeth "chip-pulpitis" was complicated by an ascending pulpitis which developed in the course of advanced resorption of alveolar bone and root (KALNINS, 1958). Only two of the eighteen cases with pulpitis had shown discomfort upon application of cold water.

Another feature of the inflamed pulp was the resorption of chips (Figs. 6, 8). The resorption took place chiefly with the presence of giant cells and macrophages. In other inflamed pulps the osteoclasts had disappeared and in their place the lacunae were occupied by neutrophils (Fig. 8).

An encapsulation of the dentine fragments was observed in ten cases. Large dentine particles were sometimes embedded in "callus" (Figs. 9, 11). Chip conglomerates intermixed with capping paste were demarcated from the remaining pulp by means of hard tissue or a fibrous capsule (Fig. 10). Here inflammation was less pronounced or absent. Where advanced pulpitis was present no encapsulation was observed.

The order and position of bridge formation is related to the extent and degree of inflammation. In cases with advanced pulpitis, bridge formation was incomplete (Fig. 14) or absent (Fig. 1). In some cases with a circumscribed pulpitis of low severity, the dentine bridge was formed above the focus of inflammation (Figs. 3, 5).

Also non-inflamed pulps with dentine fragments showed disturbances in wound healing. Bridge formation was imperfect and consisted in part of dentine and in part of "callus" with scattered embedded fragments. Only four teeth with single fragments in the superficial portion of the pulp lesion had formed regular bridges. In other teeth where conglomerates of small chips mixed with paste were present, the bridges also showed defects without "callus" formation (Fig. 12). In some cases where chips were present, even after a long post-operative period, pulpal healing occurred without the formation of a bridge or any other calcified structure (Fig. 13). There was no inflammation nor encapsulation of the chips into a "callus" in teeth of adults with senile involution of the pulps.

DISCUSSION

The presence of inflammation of various degrees, resorption and encapsulation of dentine chips and fragments in exposed pulps following capping shows a foreign body reaction. The severity of the inflammation seems to be proportional to the total surface area of all dentine chips present. Thus appreciable amounts of necrotic material derived from numerous small chips generally produced diffuse pulpitis or

abscess. In contrast, cases with a few large dentine chips developed mostly circum-scribed pulpitis with prevalence of chronic inflammatory cells.

Therefore, as occurs in tooth extraction, where the removal of fragments of broken alveolar bone becomes necessary, all dentine chips resulting from pulp amputation or capping should be removed from the area of operation. It is possible that the contradictory reports offered by several workers (CASTAGNOLA, 1953; GLASS and ZANDER, 1949; VAN HUYSEN and BOYD, 1953; KALNINS, 1957) for the effectiveness of various pulp capping agents may largely be occasioned by the presence or relative absence of dentine chips left in the pulp while operating.

Moreover, it should be recognized in practice that "dentine chip pulpitis" may be asymptomatic (KALNINS, 1957), since an advanced pulpitis may occur without any significant dilation of blood vessels (Figs. 1, 7). It can be present also in teeth which radiographically show dentinal bridges (Fig. 3).

The complete removal of dentine chips and fragments from the operation site is difficult. It may be necessary to remove the entire roof of the pulp chamber in order to avoid undercuts and retention of chips. With such wide exposure of the pulp many of the dentine chips are flushed away through copious bleeding.

Wide opening of the pulp is especially important in cases of carious exposures with or without manifest symptoms of pulpitis. As in other wounds, pulpal bleeding is essential to wash away all detritus, such as food remnants, clumps of bacteria, pus from localized abscesses, necrotic tissue and carious or non-carious dentinal fragments forced into the pulp during the operation.

TABLE 1.   THE EFFECT OF INSERTED DENTINE CHIPS AND FRAGMENTS ON THE
HEALING OF EXPOSED PULPS

Number of teeth	Cases with small chronic inflammatory foci	Cases with diffuse pulpitis or abscess	Cases with encapsulated chips	Cases of absence or defective bridge formation due to dentine fragments		Cases with absence of reaction to dentine chips
				With inflamed pulps	With non-inflamed pulps	
Permanent 24	4	9	(3)*	(7)*	6	5
Primary 22	7	9	(7)*	(6)*	5	1
Total 46	11	18	(10)*	(13)*	11	6

* These cases were in combination with other ones and therefore should not be counted in the total.

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FIG. 1. Third molar tooth, showing the formation of two abscesses associated with dissemination of numerous small dentine chips in the pulp. One of the abscesses (x) is located in the site of the pulpal wound; the larger and deeper one (xx) is at the bifurcation (bc). There are no signs of bridge formation in spite of 5 weeks postoperative period. Note the absence of hyperaemia. CaS = remnants of the paste. pr = pulpal roof. Bock staining. ×18.

FIG. 2. Higher magnification of an area of the deeper abscess marked as (xx) in Fig. 1. The dentine chips (f) are surrounded mostly by neutrophils. Bock staining. ×270.

FIG. 3. Circumscribed pulpitis below the bridge which resulted from insertion of chips and fragments into the pulp. In spite of inflammation (x), the pulp produced a dentine bridge (br) in the site of the wound. (Primary canine 5 months postoperatively). Haematoxylin and eosin. ×36.

FIG. 4. Higher magnification of an area of the inflammatory focus (x) from Fig. 3, showing the dentine chips (f) lymphocytes, and macrophages (indicated by arrows). Haematoxylin and eosin. ×387.
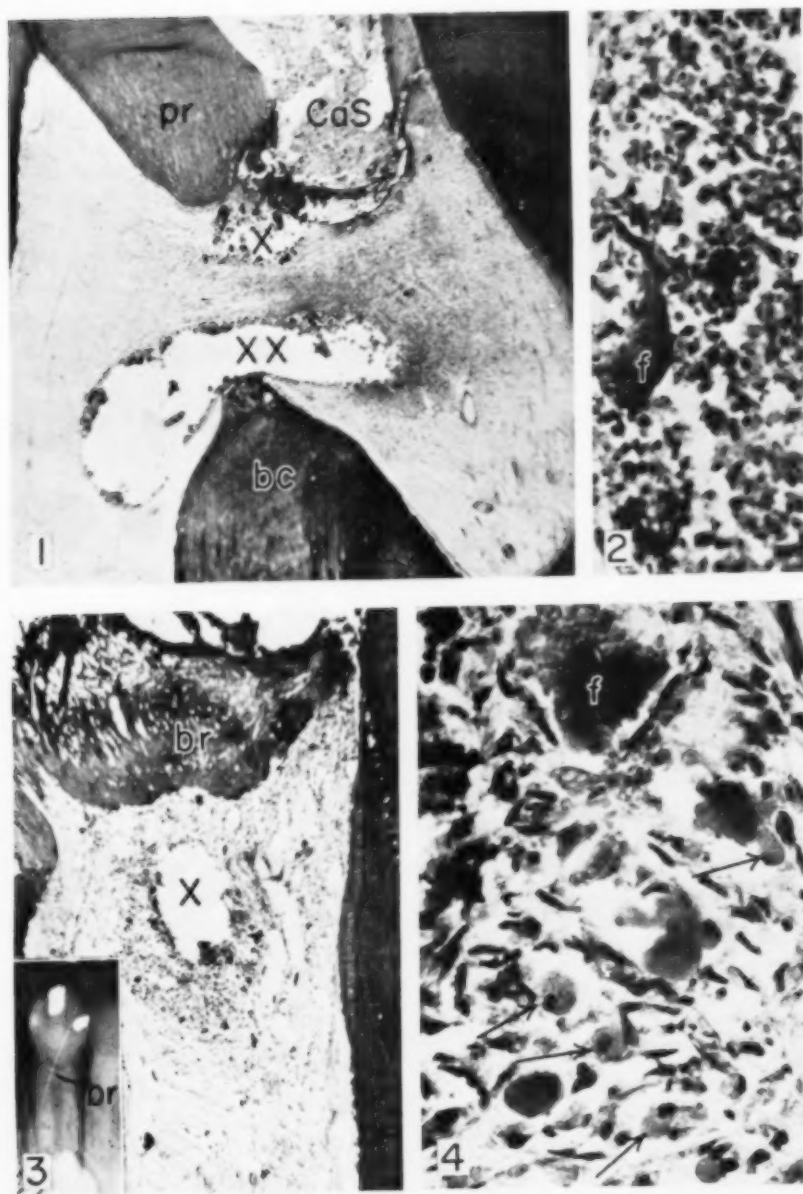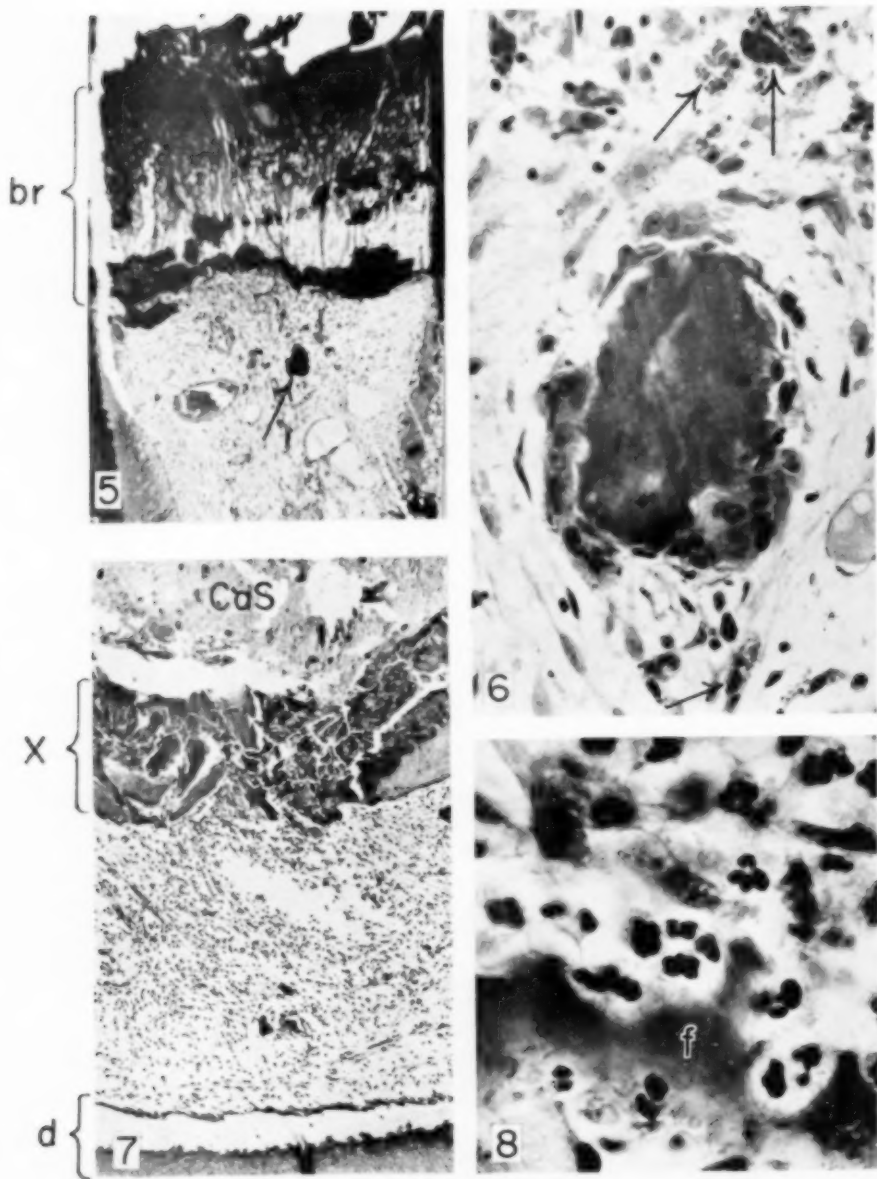
PLATE 1

PLATE 2

FIG. 5. A large dentine fragment (indicated by arrow) is located below the bridge (br) which consists of fibrillar dentine and dystrophic calcifications. The area of the pulp between the fragment and the bridge shows minor infiltration by inflammatory cells. The remaining pulp shows hyperaemia. (Primary canine 4½ months post-operatively). Bock staining. ×40.

FIG. 6. Higher magnification from Fig. 5 of the pulp area containing the dentine fragment. The fragment is undergoing resorption in the presence of giant cells. The adjacent pulp shows macrophages (indicated by arrows), lymphocytes and fibroblasts. Bock staining. ×302.

FIG. 7. Diffuse pulpitis in the presence of dentine chips and fragments (x) at the site of the pulpal wound. (Primary molar 2 months post-operatively). CaS= remnants of the paste. d=dentine. Haematoxylin and eosin. ×54.

FIG. 8. Higher magnification of pulp area (x) in Fig. 7 containing dentine chips and fragments. The fragment (f) shows resorption. The lacunae are now occupied by neutrophils. Hansen staining. ×450.

FIG. 9. Large dentine fragments (indicated by arrows) embedded in "callus" (x). (Primary canine 7 months post-operatively). Haematoxylin and eosin. ×54.

FIG. 10. Numerous dentine chips mixed with paste are seen embedded in a capsule which consists of both hard substance (b) and fibrous tissue (f). CaS=paste. Appearance of "reticular atrophy" of pulp is a shrinkage artefact produced during histological preparation. (Primary canine 7 months post-operatively). Haematoxylin and eosin. ×40.

FIG. 11. Coronal portion of pulp (a) shows dentine chips embedded in callus. Apical portion (b) shows large dentine fragments surrounded by plasma cells and macrophages. Inflammation is absent from the remainder of the pulp. (Primary canine 7 weeks post-operatively). Haematoxylin and eosin. ×54.

PLATE 3

Plate 4
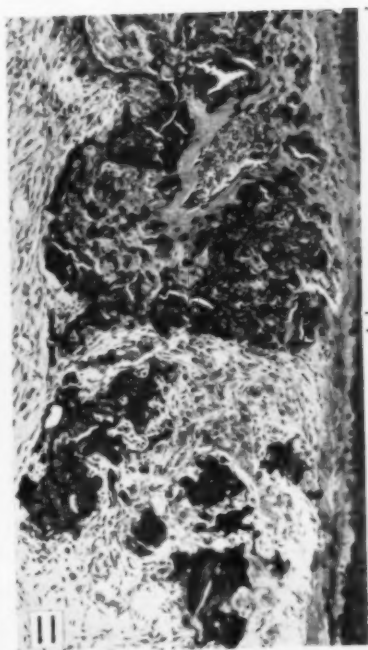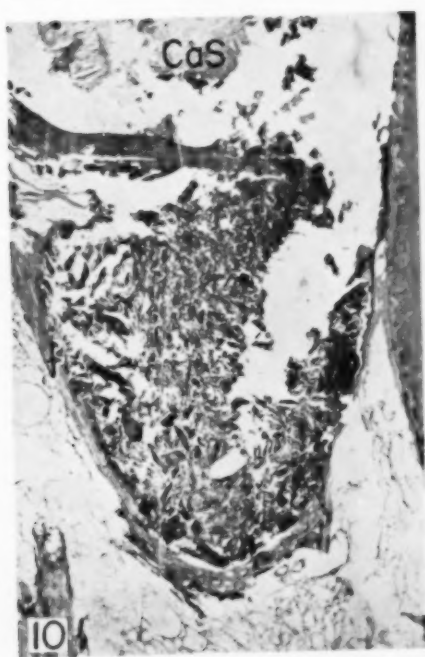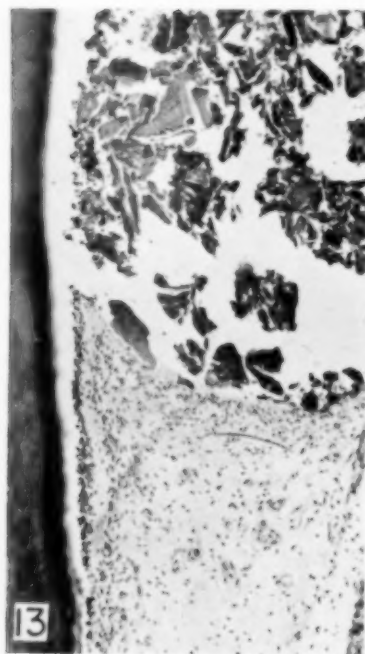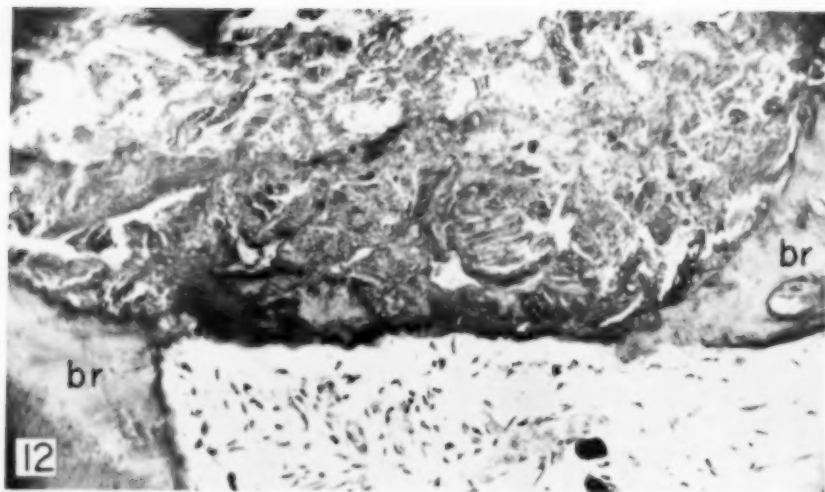
FIG. 12. The defect in the bridge (br) due to presence of a conglomerate of small dentine chips mixed with paste. (Premolar 4 months post-operatively). Haematoxylin and eosin. ×250.

FIG. 13. Healed pulp wound without formation of dentine bridge in the presence of numerous dentine chips. The pulp shows normal structure. (Primary molar 4 months post-operatively). Hansen staining. ×90.

FIG. 14. Granulomatous pulpitis provoked by dentine chips (indicated by arrows). Below the inflamed area, a rudimentary bridge (br) consists partly of irregular dentine and partly of scar tissue. Small dentine chips and hyperaemia are also present below the bridge. (Primary canine 4 months post-operatively). Haematoxylin and eosin. ×36.

QUANTITATIVE STUDIES OF THE BACTERIAL FLORA OF THE PERIODONTIUM IN RICE RATS*

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Abstract—Eighteen 21-week old rice rats were scored for periodontal disease according to the method of GUPTA and SHAW (1956). The molar teeth and periodontium of one side of the heads were weighed, and ground in broth. Dilutions determined in preliminary trials were plated in 0·1 ml amounts on each of five plates of the following media: horse blood agar, Na azide agar, nutrient agar, EMB agar, Rogosa SL agar, serum tellurite agar. The blood agar was incubated anaerobically for 5 days, other media aerobically for 3 days. Colony counts were made at $10 \times$ magnification with a stereoscopic microscope. Total counts from growth on blood agar incubated anaerobically ranged from $3 \cdot 6 \times 10^7$ to $3 \cdot 6 \times 10^{10}$ per total sample. Counts averaged $3 \cdot 4 \times 10^8$ for twelve animals with periodontal scores of 57 or less, and more than $1 \cdot 6 \times 10^{10}$ for six animals with scores exceeding 100. Enterococci were the most numerous organisms, counts ranging from $2 \cdot 0 \times 10^6$ to $3 \cdot 5 \times 10^{10}$. High counts, averaging $1 \cdot 4 \times 10^{10}$, were associated with high periodontal disease scores. *Actinobacillus* counts ranged from $1 \cdot 0$ to $5 \cdot 6 \times 10^8$ for the four rice rats with highest periodontal scores. Except for one animal, remaining *Actinobacillus* counts averaged $6 \cdot 9 \times 10^6$. The only other regularly occurring organisms were diphtheroids ranging in counts from $3 \cdot 0 \times 10^6$ to $7 \cdot 3 \times 10^7$ and unrelated to periodontal disease. Staphylococci, coliforms and lactobacilli each appeared on plates from over half the animals, usually in the ranges 10^6–10^7, 10^4–10^5, 10^3–10^6, respectively. *Fusobacterium*, although observed only twice, numbered $1 \cdot 2 \times 10^8$ in one animal.

MANY investigations of numbers of indigenous oral organisms in experimental animals have dealt with lactobacilli in relation to caries. In addition, data on numbers of streptococci in the hamster oral cavity have been published by ROGOSA, JOHANSEN and DISRAELY (1957). WAKEMAN *et al.* (1948) described a method of obtaining total counts of oral bacteria in the cotton rat. On the basis of counts from swabs, figures have been reported for numbers of a few groups in the rat oral cavity, namely *Veillonella* (ROGOSA, 1956), diphtheroids (ROGOSA, SHIOTA and DISRAELY, 1957), streptococci and coliforms (ROSEN, RAGHEB, HOPPERT and HUNT, 1959). No previous attempt has been made to survey quantitatively the total oral flora of an experimental animal. Such an attempt seemed feasible for the rice rat in view of the character of the flora as previously reported (MACDONALD, SOCRANSKY

and SAWYER, 1959). It also seemed desirable as an approach to elucidating the character of changes which might occur in the flora incidental to periodontal disease in this species of rodent.

METHOD

Rice rats from both sexes, 21 weeks old, were killed, decapitated and the skins removed from their heads. The heads were divided sagitally; one half was frozen in solid carbon dioxide and the other half was used to determine periodontal disease scores (GUPTA and SHAW, 1956). On the basis of the total scores for both soft tissue lesions and bone loss, eighteen animals were selected for quantitative bacteriological studies. Six had scores considered to be low (24–36), indicative of negligible periodontal disturbance, six were classified as medium scores (46–57), and six as high scores (106–159).

The frozen half of each head was thawed, and the molar teeth and associated periodontal structures of both jaws were removed *en masse* by sterile Rongeur forceps, weighed, diluted 100-fold on a w/v basis in heart infusion broth, and ground in a glass tissue grinder. Serial 10-fold dilutions of the resultant suspensions were prepared in heart infusion broth.

One-tenth ml of appropriate dilutions was plated on media as follows: heart infusion agar with 10% horse blood (10^{-5}, 10^{-6}, 10^{-7}), sodium azide agar with 10% horse blood (10^{-6}, 10^{-7}), nutrient agar (10^{-5}, 10^{-6}, 10^{-7}), EMB agar (10^{-3}, 10^{-4}, 10^{-5}), Rogosa SL agar (10^{-3}, 10^{-4}, 10^{-5}) and serum tellurite agar (10^{-3}, 10^{-4}). All media were Difco products. Five plates were prepared at each dilution and the dilutions were selected on the basis of preliminary experiments to provide at least one set of plates suitable for counting colonies. The heart infusion blood agar plates were incubated anaerobically for 5 days, all others aerobically for 3 days at 37°C. Colonies were counted using a stereoscopic microscope (magnification ×10).

The heart infusion blood agar was used as a best estimate for the total count of cultivable organisms and to count colonies of *Fusobacterium*. The sodium azide blood agar was used for counting enterococcus colonies, nutrient agar for *Staphylococcus* and *Actinobacillus* colonies, EMB agar for coliforms and late lactose fermenters, Rogosa SL agar for lactobacilli and serum tellurite agar for diphtheroids. Gram stained smears were made frequently to verify the identity of colonies being counted. All of the above types of organisms were identified by biochemical tests in earlier studies as components of the rice rat oral flora (MACDONALD, SOCRANSKY and SAWYER, 1959).

RESULTS

The counts in the following description are expressed as means for five plates multiplied by the appropriate dilution factor to arrive at an estimate of the total organisms of each type in each sample. The samples represented half the molar periodontium and weighed on the average 66 mg (range 40–90 mg).

A relationship appeared to exist between colony counts and periodontal disease scores in the case of the total counts (determined from blood agar plates incubated anaerobically), the counts for enterococci, and possibly the counts for *Actinobacillus* species. The data are recorded in Table 1.

TABLE 1. COLONY COUNTS* (TOTAL, ENTEROCOCCI AND ACTINOBACILLI) AND PERIODONTAL DISEASE SCORES FOR EIGHTEEN RICE RATS

Periodontal disease scores	24	28	31	32	33	34	46	48	49	50	51	57	108	114	130	138	150	159
Total count (blood agar)	12·0	6·6	62·0	3·6	19·0	14·0	38·0	14·0	10·0	190·0	18·0	19·0	680	1100	1700	3550	1500	1100
Total count (summation)†	12·0	6·4	35·0	2·0	16·0	20·0	12·0	9·0	7·0	170·0	9·2	3·8	610	750	1500	3510	1400	810
Enterococci	5·2	2·6	34·0	0·2	11·0	9·8	11·0	8·6	0·8	150·0	4·4	1·2	600	740	1500	3500	1400	790
Actinobacilli	0·86	0·62	0	0·10	1·7	3·2	0·14	0·02	0	14·0	0·30	0·40	1·4	0·18	26·0	10·0	56·0	11·0

* Counts ×10⁻³.
† Counts derived by summing mean counts on all media except blood agar.

The total count as estimated from the blood agar plates averaged $3·4 \times 10^8$ for twelve animals with periodontal disease scores of 57 or less. The six animals whose scores exceeded 100 had total counts averaging $1·6 \times 10^{10}$. The difference is highly significant as determined by an analysis of variance (Table 2). Significant differences did not occur between the twelve animals in the lower group but did occur between animals with high scores, although the differences were not related to the scores ($D=QS_{\bar{x}}=204·9$ where $Q=$upper 5 per cent point in Studentized range and $S_{\bar{x}}=$standard deviation due to plate count variation).

Evidence that the blood agar plate counts are in fact estimates of the total of cultivable species can be found in a comparison of the plate count totals and the totals derived by summation of mean counts on all media except blood agar (Table 1). It can be seen that there is good agreement between the totals computed by both methods. The difference between the counts is less than two-fold in fifteen of eighteen instances and the largest difference is five-fold.

The counts of enterococci obtained from sodium azide blood plates averaged $2·0 \times 10^8$ for twelve rice rats with the lowest counts and $1·4 \times 10^{10}$ for the six with the highest counts (Table 1). The difference is highly significant (Table 3) and shows that the counts of enterococci are related to periodontal disease scores. Again it can be seen that low score animals did not differ significantly from each other in terms of enterococcus counts. The high score animals, however, did differ significantly within the group, though without relation to periodontal disease scores ($D=244·0$).
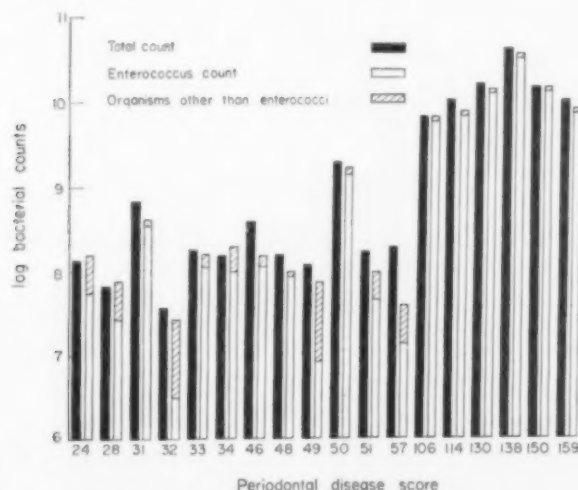
FIG. 1. Bacterial counts and periodontal disease score.

It is evident that the increased bacterial concentrations in advanced periodontal disease in the rice rat are attributable mostly to the enterococci. This is illustrated in Fig. 1 where total counts and enterococcus counts are plotted in relation to

periodontal scores. The difference between the total counts and enterococcus counts remains relatively small, while both are increased in the presence of severe periodontal destruction. Expressed in another way, the enterococci on the average constituted 40 per cent of the total count in animals with low scores and 85 per cent of the total in the animals with high scores for periodontal disease.

TABLE 2. ANALYSIS OF VARIANCE OF TOTAL COUNT DATA

Sources of variation	Degrees of freedom	Sums of squares	Mean squares
Total	$85^* - 1 = 84$	2,802,602,170	
Rats	$17^* - 1 = 16$	2,747,496,490	171,718,530
Plate count error	68	55,105,680	810,377

* One high count animal (p.d. score 138) omitted from computation. Plates too crowded for accurate count.

$F = \dfrac{171,718,530}{810,377} = 211$ (highly significant).

Significant difference $(D) = 204 \cdot 9$.

TABLE 3. ANALYSIS OF VARIANCE OF THE ENTEROCOCCUS COUNT DATA

Sources of variation	Degrees of freedom	Sums of squares	Mean squares
Total	$85^* - 1 = 84$	2,117,530,500	
Rats	$17^* - 1 = 16$	2,039,340,200	127,458,762·5
Counts	68	78,190,300	1,149,857·3

* One high count animal (p.d. score 138) omitted from computation. Plates too crowded for accurate count,

$F = \dfrac{127,458,762 \cdot 5}{1,149,857 \cdot 3} = 110 \cdot 8$ (highly significant).

Significant difference $(D) = 244 \cdot 9$

Actinobacillus colony counts ranged from $1 \cdot 0 \times 10^8$ to $5 \cdot 6 \times 10^8$ for the four rice rats with highest periodontal disease scores. Except for one animal with a similar count ($1 \cdot 4 \times 10^8$) the remaining counts averaged $6 \cdot 9 \times 10^6$. In two instances no organisms in this category were detected. Since the lowest dilution plated was 10^{-5}, this finding would be compatible with an occurrence in these two samples of as many as 10^5 actinobacilli. Analysis of variance indicates that the differences between rats were significant. The significant difference ($D = 2 \cdot 5$) indicated that the counts for the four animals with highest periodontal disease scores were distinguishable statistically

from the remainder (with the exception of the one animal referred to above). The results suggest inconclusively that *Actinobacillus* counts may increase as a late phenomenon in periodontal disease in the rice rat.

TABLE 4. COLONY COUNT DATA ON ORGANISMS FOR WHICH COUNTS DID NOT VARY IN RELATION TO PERIODONTAL DISEASE

Organism	No. of times detected	Lowest dilution plated	Mean (excluding negative results)	Range (excluding negative results)
Aerobic diphtheroids	18	10^{-3}	$2 \cdot 7 \times 10^7$	$3 \cdot 0 \times 10^5 - 7 \cdot 3 \times 10^7$
Staphylococci	12	10^{-3}	$1 \cdot 9 \times 10^6$	$6 \cdot 0 \times 10^2 - 1 \cdot 1 \times 10^6$
Paracolon bacilli	13	10^{-3}	$1 \cdot 4 \times 10^6$	$1 \cdot 0 \times 10^4 - 1 \cdot 1 \times 10^7$
Coliforms	14	10^{-3}	$5 \cdot 3 \times 10^6$	$4 \cdot 0 \times 10^3 - 3 \cdot 7 \times 10^7$
Lactobacilli	9	10^{-3}	$1 \cdot 3 \times 10^6$	$2 \times 10^3 - 6 \cdot 4 \times 10^6$
Fusobacteria	2	10^{-6}	$6 \cdot 0 \times 10^7$	$1 \cdot 6 \times 10^5 - 1 \cdot 2 \times 10^8$

Aside from enterococci and actinobacilli, no other categories counted appeared to be related quantitatively to periodontal disease. The data are summarized in Table 4. Diphtheroids were regularly recovered and counts varied significantly between animals but in an apparently random fashion without relation to periodontal disease scores. The counts for staphylococci, coliforms, paracolon bacilli and lacto-bacilli were low compared to the enterococci—on the average about 100-fold less than the mean enterococcus counts among animals with low periodontal disease scores. Fusobacteria were counted by detection of their distinctive colony morphology on blood agar. They were detected and counted from two samples only. Since they were counted on plates showing heavy growth of the total spectrum of cultivable species and since the countable plates were diluted at least to 10^{-6}, the failure to detect *Fusobacterium* regularly cannot be considered to indicate relatively rare occurrence or even occurrence in only low numbers. Of interest is the fact that *Fusobacterium* counts for one sample averaged $1 \cdot 2 \times 10^8$.

DISCUSSION

It is clear from these findings that periodontal disease in the rice rat is not accompanied by an over-all proliferation of the bacterial flora. Though there is an increase in total count, it is attributable to specific components of the flora (entero-cocci, and possibly actinobacilli) rather than a proportional increase in all species. It has seemed likely that in human periodontal disease the flora as a whole proliferates (HEMMENS and HARRISON, 1942; ROSEBURY, MACDONALD and CLARK, 1950). SCHULTZ-HAUDT and SCHERP (1955), however, reported a higher proportion of hyaluronidase-producing viridans streptococci in samples from the mouths of persons with marginal gingivitis than in samples from individuals free from gingivitis. The findings in the present study, though obviously not applicable to man, suggest the need for comparable quantitative studies in man.

It is of interest to ask whether enterococci have special significance in the aetiology of rice rat periodontal disease or whether increase in the numbers of these organisms is a phenomenon of advanced periodontal disease attributable to change in the environment. Scores as high as 57 occurred without evidence of increase in the enterococcus counts. Animals with such scores had definite lesions and it is therefore apparent that measurable increase in the numbers of enterococci is not a prerequisite to the development of periodontal disease. This leads to the conclusion that increase in numbers of enterococci is probably associated with changes in environment incident to developing disease. It does not, however, permit an assumption that the increase in enterococci is without significance in the subsequent course of periodontal disease. In this connexion it is of interest that GUPTA, AUSKAPS and SHAW (1957) inhibited the development of periodontal lesions with penicillin. Studying the enterococcus counts in four rice rats on penicillin-containing diets we found the average counts about 100-fold less than in animals with low periodontal disease scores and no penicillin. *In vitro* tests indicated also that the enterococci were highly sensitive to penicillin. Further studies with other antibiotics may throw additional light on the significance of enterococci and other organisms in relation to the pathogenesis of periodontal disturbances.

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ELECTRON MICROSCOPY OF THE DEVELOPING CARTILAGENOUS EPIPHYSIS

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Abstract—Thin sections of developing cartilagenous epiphyses of phalangeal bones from mice have been observed under the electron microscope. The first degenerative changes in the chondrocytes seem to occur as dispersion and destruction of the cytoplasmic organelles. The fibrils in the cartilage matrix do not appear in definite bundles but are arranged in a fine network. Although fibril cross-banding is difficult to observe, clear 220Å striations are occasionally found. The mineralized cartilage matrix contains an unknown amorphous or finely granular substance in addition to the fibrillar component. In the later stages of the mineralization, the calcified areas are delimited by a dense line.

INTRODUCTION

CARTILAGE growth in the epiphyseal region has been studied extensively by optical microscopy (FELL, 1925; BLOOM and BLOOM, 1940). During recent years especially, histochemical investigations (PRITCHARD, 1952; FOLLIS and BERTHRONG, 1949) have made it possible to trace the complicated changes which take place, and have also assisted in clarifying the nature of some of the structural elements themselves. Further detailed information has been brought forth through electron microscopy (RANDALL *et al.*, 1952; MARTIN, 1954; JACKSON, 1954; LITTLE and PIMM, 1957; SCOTT and PEASE, 1956; ROBINSON and CAMERON, 1956, 1957; CAMERON and ROBINSON, 1958). The entire structure of the cartilagenous epiphysis has not yet been examined by this latter method in detail, and some parts still remain to be observed.

The present paper is a survey of the developing cartilagenous epiphysis in the mouse. Although the observations are still relatively incomplete, some findings have been obtained which should be added to the previous observations made by other researchers.

MATERIAL AND METHODS

The specimens used were developing phalangeal bones from mice ranging in age from 1 to 8 days. The bones were dissected from anaesthetized animals and placed immediately in a 1 % osmium tetroxide–1 % potassium dichromate mixture (DALTON and FELIX, 1955). They were left in the fixative for 18 hr at 4°C. After rinsing, the specimens were dehydrated in alcohol and embedded in a mixture of butyl and methyl methacrylates according to the usual methods. None of the samples were subjected to preliminary decalcification.

Microtomy was carried out with either a Porter–Blum microtome or a modified Spencer microtome which was equipped for thermal advance of the specimen and

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bypassing of the knife on the return stroke (NYLEN and HOLLAND, 1957). Glass knives were used in both instances.

In order to select the portions of the blocks from which thin sections were to be made, relatively thick sections were cut for inspection under the optical microscope. After the desired area was thus located the block was reduced in size so that the final truncated pyramid presented a surface to be cut which was approximately 1/2 mm square.

The ribbons of sections were picked up on specimen screens previously covered with carbon substrates, and observed without removal of the embedding medium.

In order to bring out the structure of the matrix fibrils in more detail, some sections were stained with phosphotungstic acid or shadowed with tungsten oxide. The staining was done by floating the sections on the surface of a 10% aqueous solution of phosphotungstic acid for 2 hr. After water-washing the ribbons were placed on specimen screens for observation. Although the sections were decalcified, due to the acidity of the solution, the matrix fibrils were brought out more clearly through this treatment. When samples were to be shadowed, the methacrylate was dissolved by immersing the sections on the specimen screens in amyl acetate for 1 hr. Since tungsten oxide is amorphous these specimens could not only be examined under the electron microscope but they could also be subjected to limited area electron diffraction.

Some sections were decalcified in place on the specimen screens by immersion for 30 min in 2% hydrochloric acid, following removal of the methacrylate.

RESULTS

The X-ray micrograph of Fig. 1, which was obtained from the epiphyseal region of a developing phalangeal bone of a 4-day old mouse, is shown as an aid in visualizing the location of the various areas which are illustrated in the electron micrographs. At this point in development, the cartilagenous epiphysis has become well defined and has differentiated into several zones which are termed, from the articular surface to the diaphyseal side, epiphyseal, flattened-cell and hypertrophied (FELL, 1925). Toward the diaphyseal side of the hypertrophied zone, the cartilage cells are undergoing degeneration and mineralization of the matrix is taking place (degenerated zone). Much of the diaphyseal portion of the cartilage model has been removed and endochondral bone formation has commenced on the remnants of the cartilage bars.

The articular surface of the cartilagenous epiphysis from a 5-day old mouse is shown in Fig. 2. The space in the upper part of the picture is the edge of the synovial cavity. The chondrocytes with the neighbouring cartilage matrix impart a scalloped contour to the cavity, which appears to be completely empty. The chondrocytes depicted in this micrograph are round or ovoid, but in many instances they appear spindle-shaped. They have many short cytoplasmic processes, and in the cytoplasm the endoplasmic reticulum consists for the most part of typically parallel membrane pairs. In some areas, however, the membranes are widely separated, giving the impression that the reticulum is dilated. The matrix in the subarticular surface area is made up of closely-packed minute fibrils (Fig. 3). They are arranged in an irregular network and do not appear in definite bundles.

The epiphyseal zone of the cartilage is shown in Fig. 4. The chondrocytes are irregularly circular, and typical endoplasmic reticulum and many mitochrondria are evident in their cytoplasm. The dilated type of endoplasmic reticulum can also be observed. The matrix in this epiphyseal zone, as shown in Fig. 3, resembles that in the sub-articular surface area (Fig. 3). In this picture, however, fairly clear striations can be observed on some of the fine fibrils. The thickness of these fibrils is 200–400Å and the periodicity of their striation is about 220Å.

The structure of the flattened-cell zone of the cartilagenous epiphysis is shown in Fig. 6. In this zone the chondrocytes are flatter and closer together, and many long cytoplasmic processes and loops can be seen. Adjacent cells are sometimes joined together by narrow cytoplasmic bridges. In the cytoplasm there are numerous vacuoles of various sizes which contain matter of low density. The remainder of the cytoplasm is taken up by the endoplasmic reticulum and mitochondria. These organelles appear closely packed. Between the chrondrocytes and the fibrillar matrix wide spaces are found which are seldom seen in the epiphyseal zone. These spaces are occasionally filled with more sparsely arranged fibrillar material (Fig. 7).

The hypertrophied and degenerated zones are illustrated in Fig. 8. In the hypertrophied zone the cartilage cells increase in size. Their cytoplasm assumes a structure similar to that of the cells in the epiphyseal zone, but a more closely packed parallel arrangement of the endoplasmic reticulum is clearly seen.

In the large degenerated chondrocytes, the cytoplasmic organelles are more widely scattered than in the cells of the other zones. The endoplasmic reticulum decreases in quantity and becomes quite irregular in arrangement. Relatively opaque masses of undetermined identity can be seen. Many of them have some similarity to the dilated type of endoplasmic reticulum. Destruction of the mitochrondria seems to start after the endoplasmic reticulum has begun to change. Entire nuclei are often missing and seem to have been replaced by large vacuoles. At the end of degeneration the cellular remnants are scattered in the cartilage lacunae, and sometimes they disappear completely, leaving large empty spaces. The cartilage bars become thinner toward the deeper area, and finally parts of the bars disappear, resulting in a communication between neighbouring cartilage lacunae or primary bone marrow spaces. The complete destruction of the chondrocytes, however, does not always parallel the disappearance of the cartilage bars. At quite late stages, degenerating chondrocytes can occasionally be noted together with the connective tissue cells which have invaded from the primary marrow into the cavity which developed through complete destruction of the cartilage bars (Fig. 9).

In the degenerated zone there is a remarkable change in the matrix due to mineral deposition (Fig. 10). In the early stages of the mineralization small irregular-shaped clusters of fine crystals appear at various places in the cartilage bars (Fig. 11). The crystals are needle-shaped, 200–800Å long and less than 50Å wide, and do not show any regular arrangement in the clusters.

Fig. 12 was obtained from a phosphotungstic acid stained section of cartilage matrix in which mineralization is taking place. The crystals were completely dissolved out during the staining procedure. It is noticed that the area which was previously

occupied by the mineral has a dense or solidified appearance. The matrix fibrils traverse this solidified area without interruption in a somewhat parallel arrangement. There is no definite indication of a change in volume of the fibrils in the solidified area.

Fig. 13 shows a shadowed section of mineralized matrix which was decalcified on the specimen screen. An impression is given that the solidified appearance which was shown in Fig. 12 is a result of the incorporation of an amorphous or finely granular substance in the matrix.

With further mineralization the clusters of crystals gradually increase in size and fuse, finally extending over the major portion of the fibrillar matrix (Fig. 14). Some of the clusters, however, seem to remain separate without fusing. In later stages of mineralization, a dense line becomes visible at the edge of the mineralized area (Fig. 14). In decalcified sections it is brought out more clearly as a continuous structure which confines the solidified matrix (Fig. 15).

DISCUSSION

The main organelles in the cytoplasm of the chondrocytes are endoplasmic reticulum and mitochondria. The endoplasmic reticulum consists typically of parallel membrane pairs with many fine dense granules attached to their surfaces (rough-surfaced endoplasmic reticulum of PALADE, 1956). In addition, localized dilations of the reticulum are observed which resemble the globular bodies described in the odontoblasts (NYLEN and SCOTT, 1958).

WATANABE (1956) has suggested that different types of intracytoplasmic sacs (endoplasmic reticulum) are related to various phases of protein synthesis, and that the sacs become flattened at the height of protein formation. Likewise it has been claimed that the quantity of endoplasmic reticulum in some way reflects the functional status of the cell. According to the present findings it appears that while some activity may take place throughout the various zones the highest level is reached in the flattened-cell zone as evidenced by the close packing of the endoplasmic reticulum.

The significance of the vacuoles seen in the flattened-cell is still not understood. Similar unidentified vacuoles were observed by SCOTT and PEASE (1956).

Various retrograde changes in the chondrocytes can be seen in the degenerated zone. Although it is difficult to establish a definite sequence in which degeneration progresses, the first indication of a change seems to be a dispersion of the cytoplasmic organelles. These changes are followed by destruction of the organelles. Deterioration of the mitochondria appears to begin after the endoplasmic reticulum has started to change. Examination of the endoplasmic reticulum in various stages of degeneration suggests that some of the disorganized particulate matter scattered throughout the cytoplasm is in fact fragments of the reticulum itself.

JACKSON (1954) pointed out that intracytoplasmic filaments (endoplasmic reticulum) remain after the cellular degeneration and seem to be used for the succeeding bone formation. In the present studies, however, the endoplasmic reticulum did not seem to remain intact during the late stages of the degeneration.

SCOTT and PEASE (1956) observed a structure thought to be a pyknotic nucleus in a degenerating chondrocyte. In the present study, however, the nuclear material

often seemed to decrease in density with degeneration. There are also indications that the nuclei may be completely lost before the degenerated cytoplasm disappears.

It is said that degeneration of the chondrocytes occurs as a sort of intracellular oedema (WEINMANN and SICHER, 1955). The dispersion of the cytoplasmic organelles and the decrease in density of the nuclear material could be reflections of such oedematous changes, or, as was described by SCOTT and PEASE (1956), cytoplasmic hydration.

A space varying in width is noticed around the cells in the flattened-cell, hypertrophied, and degenerated zones. In some sections it appears to be empty, in others it contains some fibrillar material. SCOTT and PEASE (1956) have described an undifferentiated capsular matrix without a fibrillar component which surrounds the chondrocytes. CAMERON and ROBINSON (1958) have pointed out a moat around the cartilage cells. The space observed in the present work is believed to correspond to these structures previously described.

Varied information concerning the arrangement and structure of the cartilage matrix fibrils has been given by different investigators (RANDALL et al., 1952; MARTIN, 1954; JACKSON, 1954; LITTLE and PIMM, 1957; SCOTT and PEASE, 1956; ROBINSON and CAMERON, 1956, 1957; CAMERON and ROBINSON, 1958). Considering the findings which have been presented, arrangement and structure of the fibrils seem to be related to the regions observed, the developmental status and the type of animal used. In the present studies it was noticed that the fibrils do not appear in definite bundles and are generally arranged at random in the form of a fine network. It was found, however, that they do assume a more or less parallel arrangement in the degenerated zone. The diameter of the matrix fibrils ranges between 200Å and 400Å. In many instances it is difficult to observe a well defined periodicity in fibrils of the cartilage matrix, although some striated fibrils with a 220Å periodicity were occasionally found in shadowed sections. It is not certain that the striations are always present throughout the matrix, but it may be that they are masked by some other amorphous matrix component rather than missing.

In the degenerated zone fine crystals and amorphous or granular substance are present in the matrix. In agreement with observations by other investigators (SCOTT and PEASE, 1956; ROBINSON and CAMERON, 1956), the crystals are needle-shaped and about 50Å wide by 200–800Å long.

ROBINSON and CAMERON (1956) concluded that the mineralization of the cartilage matrix starts as a haphazard deposition of crystals without regard to the orientation of the matrix fibrils. In a more recent paper, however, they suggested that at the beginning the crystals lie parallel to the fibrils (ROBINSON and CAMERON, 1957). Although some indications of parallelism between fibrils and crystals were noted in the present material, definite conclusions about the crystal–fibril relationship could not be drawn.

The nature and origin of the amorphous or granular material which is contained in the mineralized matrix are undetermined. It is known that the calcified cartilage matrix in decalcified sections shows strong basophilia. The amorphous material just mentioned might be the component responsible for the basophilic staining reaction.

The significance of the border line surrounding the mineralized area is not known. Since it is usually seen in the later stages of mineralization, however, its appearance may indicate the termination of mineralization. If this is the case, the line could be interpreted on the same basis as the resting line in bone.

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Fig. 1. Contact microradiograph of the cartilagenous epiphysis. At the left end the synovial cavity (C) and at the right end bone formation (B) can be seen. The area between these two can be roughly divided into epiphyseal (E), flattened-cell (F), hypertrophied (H) and degenerated (D) zones. 4-day old mouse. ×126.

Fig. 2. Articular surface of the cartilagenous epiphysis. The wide space at the upper border is the edge of a synovial cavity (C). Endoplasmic reticulum (E) and mitochondria (M) are visible in the cytoplasm. 5-day old mouse. ×6480.

Fig. 3. Matrix fibrils in the same region as shown in Fig. 2. 2-day old mouse, WO₃ shadowed. ×42,750.
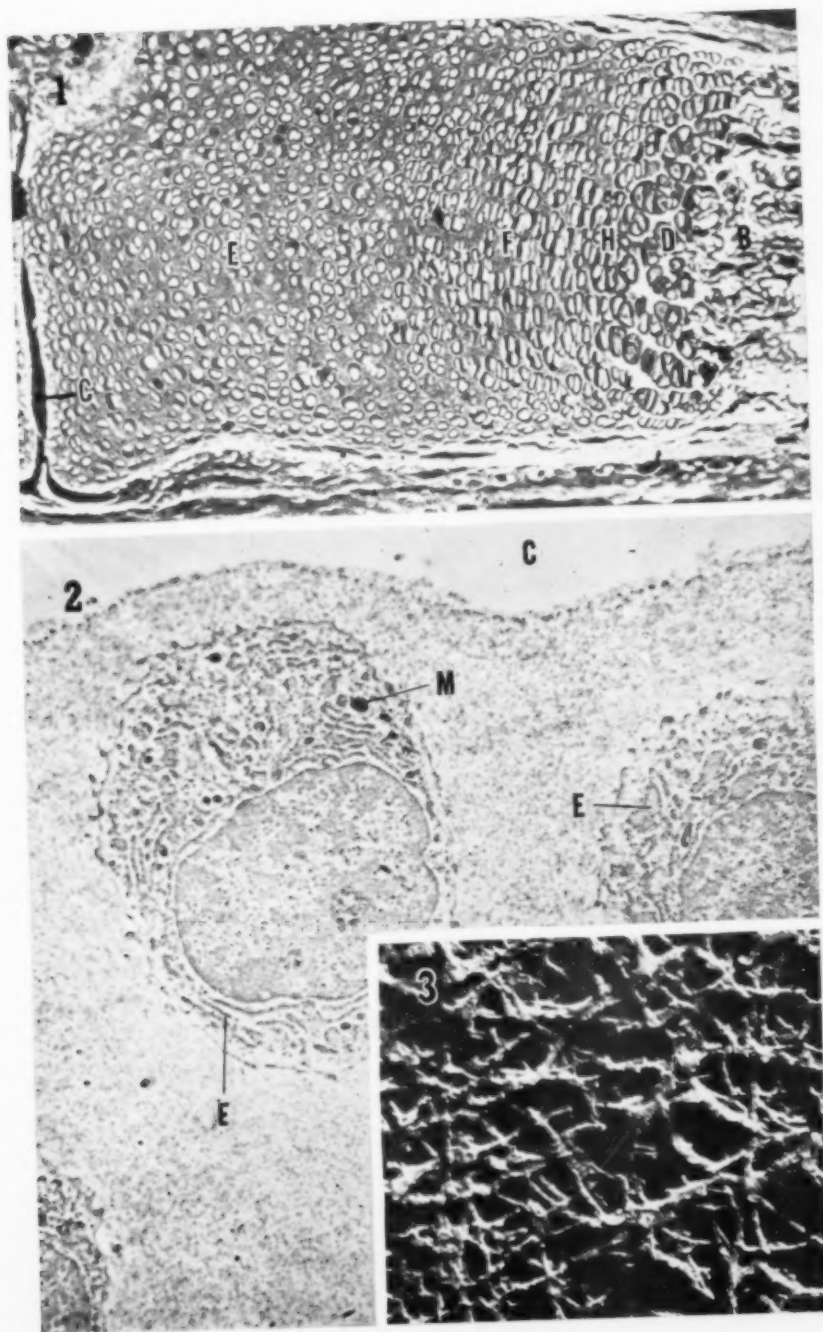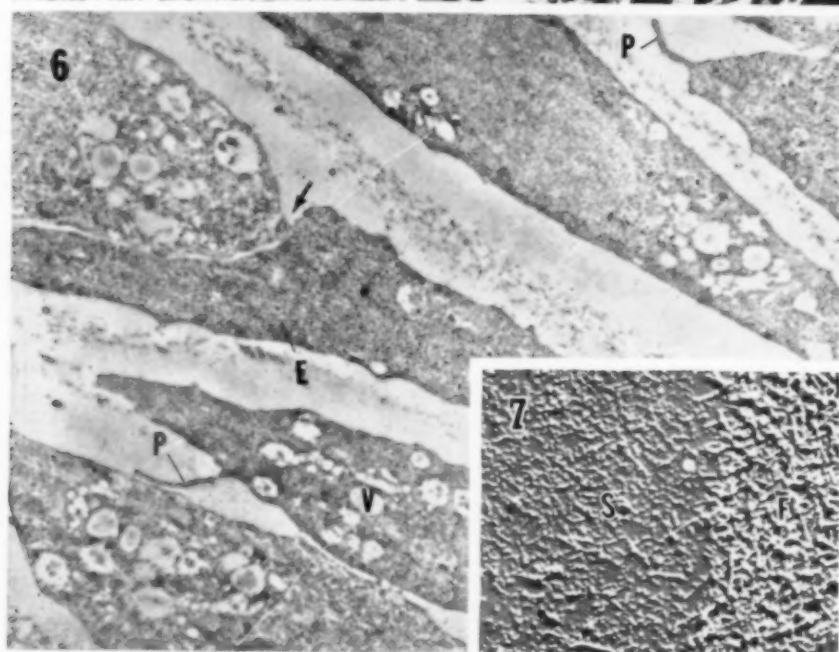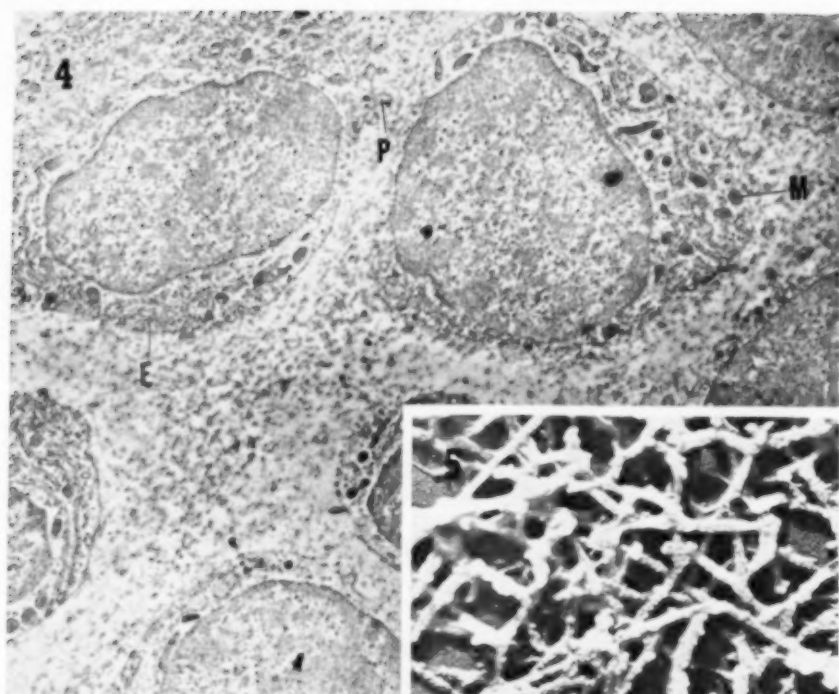
PLATE 1

Plate 2

FIG. 4. Epiphyseal zone. Short cytoplasmic processes (P), typical endoplasmic reticulum (E) and mitochrondria (M) are evident in the chondrocytes. 4-day old mouse. ×9900.

FIG. 5. Matrix fibrils in the epiphyseal zone. Fairly clear 220Å striations (arrow) are sometimes revealed in shadowed sections. 2-day old mouse, WO_3 shadowed. ×42,750.

FIG. 6. Flattened-cell zone. Vacuoles (V), endoplasmic reticulum (E), mitochondria, cytoplasmic processes (P) (arrow) are seen. 5-day old mouse. ×6480.

FIG. 7. Matrix fibrils in the flattened-cell zone. The space (S) between the cartilage matrix (F) and chondrocytes is sometimes filled with sparsely arranged fibrillar material. 5-day old mouse, WO_3 shadowed. ×10,800.

Fig. 8. Hypertrophied and degenerated zones. Close packing of endoplasmic reticulum (E) is evident in hypertrophied cell (H). Disorganization of cytoplasmic organelles and opaque masses (O) can be seen in degenerated cells (D). 5-day old mouse. ×6480.

Fig. 9. Degenerated zone. Connective tissue cell (C) which has invaded from the primary marrow is seen with degenerating chondrocytes (D). 2-day old mouse. ×10,170.

Fig. 10. Mineralization of the cartilage matrix (M). Through electron diffraction, crystalline apatite can always be detected in such an area. Cartilage lacunae (L) contain a small amount of fibrillar material. 6-day old mouse. ×6930.
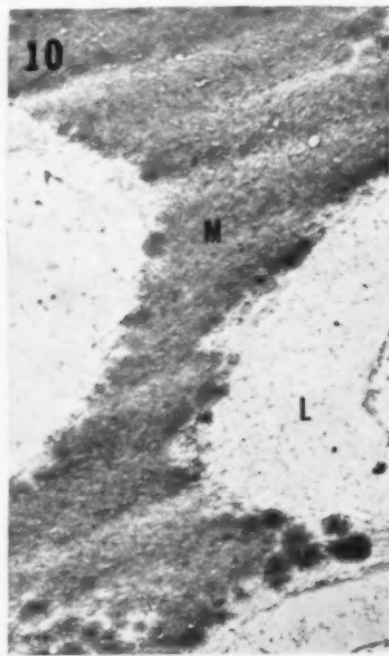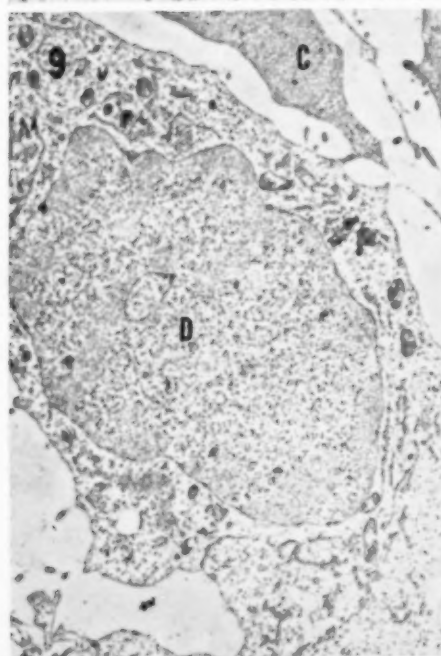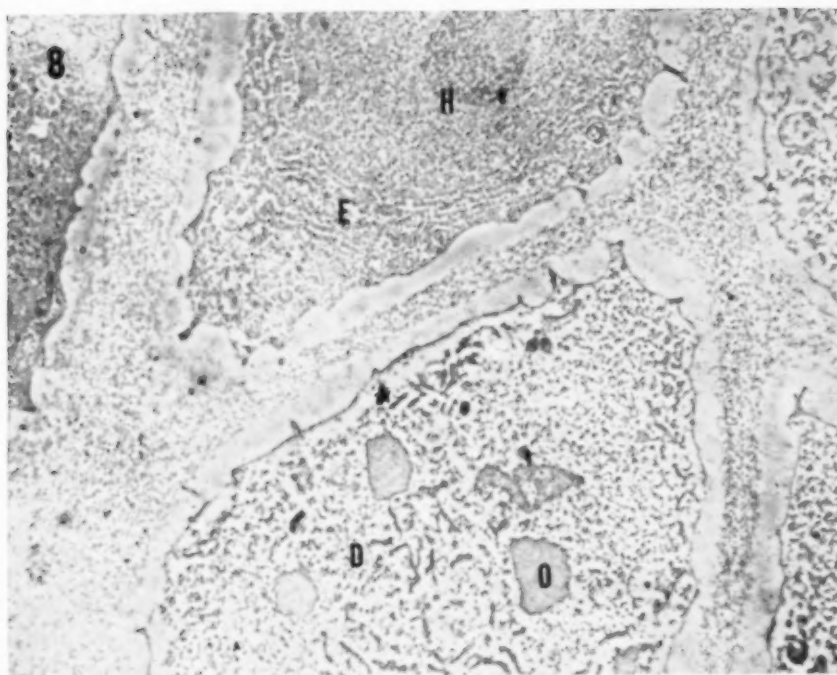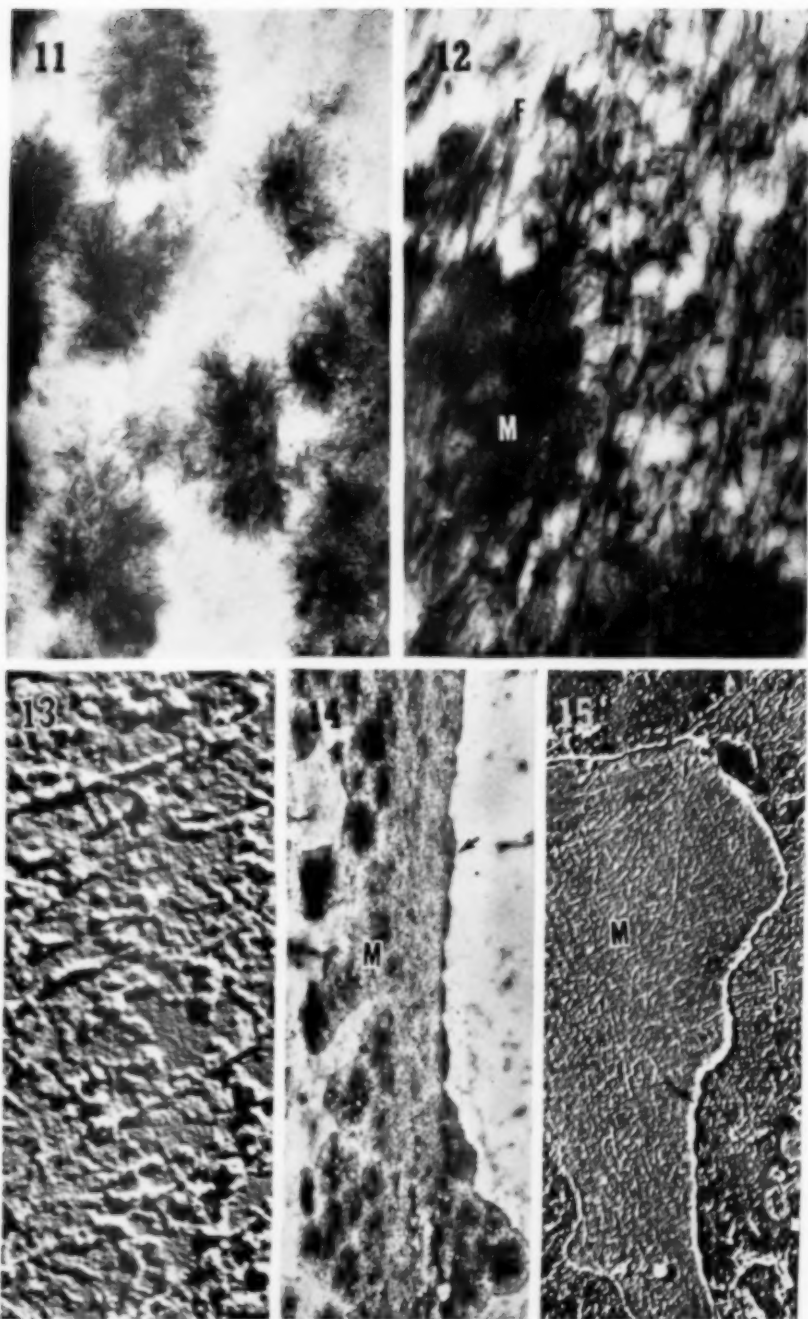
PLATE 3

PLATE 4

Fig. 11. Early stage of intracartilagenous mineralization. Many clusters of needle-shaped crystals without regular arrangement are observed. 2-day old mouse. ×45,000.

Fig. 12. Phosphotungstic acid stained section of mineralizing cartilage. The crystals were completely dissolved out, leaving matrix fibrils (F) and a dense amorphous substance (M). 3-day old mouse, stained with 10% phosphotungstic acid for 2 hr. ×42,750.

Fig. 13. Decalcified section of mineralized cartilage matrix. The matrix fibrils are embedded in the amorphous or finely granular substance. 5-day old mouse, WO_3 shadowed. ×42,750.

Fig. 14. Highly mineralized cartilage matrix. The mineralized matrix (M) is well demarcated from the unmineralized matrix by a thin bordering line (arrows). 3-day old mouse, ×8550.

Fig. 15. Decalcified section of highly mineralized cartilage matrix. The border line (arrow) between the unmineralized (F) and mineralized matrices (M) is more clearly brought up in the decalcified section. 5-day old mouse, WO_3 shadowed. ×8550.

CITRATE IN MINERALIZED TISSUES—II

THE ISOLATION FROM HUMAN DENTINE OF A COMPLEX CONTAINING CITRIC ACID AND A PEPTIDE

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Abstract—A fraction containing both citric acid and a peptide was isolated from the organic material dissolving during demineralization of human dentine with dilute acid. Quantitative amino acid analysis indicated that the hydrolysed fraction was very rich in arginine and ammonia with moderate proportions of valine, aspartic acid and leucine, while other amino acids were also present. About 30 per cent of the peptide material appeared to be a collagen fragment, the association of which with the complex may or may not be adventitious. Preliminary investigations of the structure of the complex indicated firm linkages between the peptide and citric acid.

Hartles and Leaver (1960) have used different methods of isolation to obtain from human dentine a complex containing citric acid and a peptide. Qualitative investigations indicated that the peptide was quite unlike a collagen fragment and that it was firmly bound to citric acid. The present study was made to obtain quantitative data of the amino acid composition of the peptide and to commence investigation of the structure of the complex.

EXPERIMENTAL

Isolation of the complex

The roots of human teeth were separated from the crowns as previously described (Hartles and Leaver, 1960). The dried root dentine, which probably still contained a small amount of cementum, was treated with 2N HCl until demineralization was complete, and the insoluble material removed by filtration. The filtrate was then made alkaline by the gradual addition of 10% (w/v) NaOH and the re-precipitated dentine minerals collected, dried and washed twice with distilled water. This preparation contained 0·18% N (micro-Kjeldahl) and 0·94% citric acid (Hartles and Leaver, 1960). The washed precipitate was dissolved in 2N HCl and the calcium precipitated with sodium oxalate and removed by centrifugation. The supernatant was then evaporated to dryness under reduced pressure and the resultant solids extracted three times with methyl ethyl ketone. The combined methyl ethyl ketone extracts were evaporated to dryness under reduced pressure and a brown gum was found which was shown to contain citric acid and peptide material.

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Amino acid analysis

Hydrolysis

The material was hydrolysed by refluxing for 18 hr with 6N HCl. The acid solution was evaporated to a thick syrup *in vacuo* to remove excess hydrochloric acid.

Qualitative analysis

Paper chromatography in methyl ethyl ketone/acetic acid/water (6:1:1 v/v) and propanol/ammonia/water (6:3:1 v/v) gave precisely similar results to those described by HARTLES and LEAVER (1960).

Quantitative analysis

Method. The hydrolysate was examined by quantitative chromatography on Dowex 50 ion-exchange columns by the method of MOORE and STEIN (1951) with the minor modifications described by EASTOE (1955).

The residue from hydrolysis was dissolved in pH 3·2 sodium citrate buffer and the resulting solution adjusted from below pH 0·5 to approximately pH 2·0 by the addition of 1 g of NaOH in concentrated solution. The solution was filtered through a 7 cm No. 1 Whatman paper to remove the insoluble brown residue and the paper washed with a little water. The final volume of the solution and washings was 13·0 ml.

Of this solution, 2·00 ml were added to a $100 \times 0·9$ cm resin column for analysis of the acidic and neutral amino acids and a further 2·00 ml to a $15 \times 0·9$ cm column for the basic amino acids. Fractions were analysed by the ninhydrin method of MOORE and STEIN (1948). The total ninhydrin-reactive nitrogen was determined on a small portion of the hydrolysate by the same method.

Results. The peaks of ninhydrin colour produced from the eluants of both the 100 and 15 cm columns corresponded closely in position of emergence with amino and imino acids of frequent occurrence in proteins, except for three blue peaks from the 100 cm column. These were designated I, II and III; peak I, which was small, emerged immediately before aspartic acid but too late to be ascribed to methionine sulphoxide (EASTOE, 1955); peaks II and III were larger and appeared in positions almost coincident with proline and between leucine and tyrosine respectively.

The number of gramme molecules of each amino acid per 1000 g atoms of total nitrogen was calculated and the results are given in Table 1 (column a). The hydroxyproline and proline values were not calculated owing to their peaks being overlapped by blue peaks, which would have resulted in large errors.

The total ninhydrin-reactive nitrogen (measured at 570 mμ of the filtered hydrolysate was 638 g atoms per 1000 g atoms of total nitrogen. The unreactive nitrogen of arginine and the other basic amino acids accounted for a further 298 g atoms, making a total of 936 per 1000 for the filtrate, excluding proline and hydroxyproline. Thus the brown humin material, retained on the filter, could have contained no more than 6·4 per cent of the total nitrogen. Of the 638 g atoms of total ninhydrin-reactive nitrogen only 533 g atoms (83·7 per cent) were recovered in the chromatograms as peaks, measured at 570 mμ. The reason for this discrepancy is not clear.

In column (d) (Table 1) the number of amino acid nitrogen atoms per 1000 atoms of total nitrogen has been calculated from the data in column (a). The unidentified substances were assumed to contain one nitrogen atom per molecule. Altogether, 83·1 per cent of the total nitrogen was recovered in the chromatograms, imino acids (proline and hydroxyproline) not included.

Table 1. Amino acid composition of a complex containing citric acid isolated from human dentine

	(a) Amino acids in hydrolysate of complex	(b) Amino acids in dentine collagen	(c) [=(a)—(b)] "non-collagen" amino acids of complex	(d) Gramme atoms of amino acid N in hydrolysate
Ammonia	161	7·8	153·2	161
Arginine	97	9·0	88·0	388
Glycine	53·7	61·0	(−7·3)	53·7
Aspartic acid	36·6	10·5	26·1	36·6
Valine	32·7	44·9	27·8	32·7
Glutamic acid	23·2	14·0	9·2	23·2
Unknown II	19·4	—	19·4	19·4
Leucine	19·4	4·99	14·41	19·4
Alanine	19·2	21·5	(−2·3)	19·2
Unknown III	16·3	—	16·3	16·3
Phenylalanine	12·3	2·67	9·63	12·3
Isoleucine	10·75	2·00	8·75	10·75
Serine	9·24	7·23	2·01	9·24
Tyrosine	7·50	0·43	7·07	7·50
Threonine	6·04	3·68	2·36	6·04
Lysine	2·60	4·41	(−1·81)	5·20
Methionine	1·89	0·99	0·90	1·89
Hydroxylysine	1·61	1·61	0·0	3·22
Histidine	1·49	1·01	0·48	4·47
Unknown I	1·35	—	1·35	1·35
Total	533·27	157·72	375·55	831·46

* Values are given as moles per 1000 g atoms of total nitrogen.

The entire hydrolysate of the preparation studied contained 7·5 mg of total nitrogen.

The yellow peak in the hydroxyproline position and the blue peak in the position normally occupied by hydroxylysine (an amino acid characteristic of the collagens) suggested that a proportion of collagen, or a closely related protein, was present in this preparation. To make allowance for this, the numbers of moles of "collagen" amino acids per 1000 g atoms of total nitrogen were calculated (column b) on the basis of the hydroxylysine content of the preparation and using data for human dentine collagen (Eastoe, unpublished). These numbers were then subtracted from those in column (a) to give values for the "non-collagen" amino acids in the hydrolysate (column c). Since the hydroxylysine content of collagen is small, this

method of calculation necessarily involves large errors. Nevertheless, the finding that the glycine and alanine values are both reduced to approximately zero suggests that the basis of the calculation is correct. The negative values for these amino acids have no meaning and reflect small errors in the hydroxylysine values. For the same reason, it cannot be certain that those amino acids for which very small values were obtained in column (c), e.g. methionine and histidine, are true constituents of the peptide.

Observations on the structure of the complex

When the intact citrate–peptide complex was subjected to paper chromatography in methyl ethyl ketone–acetic acid–water (6:1:1 v/v) a general streaking of faintiy ninhydrin-positive material capped by a large whitish area was observed. This characteristic result, bearing a resemblance to a white comet with faintly blue surround and tail, had previously been observed and it was concluded that the intact complex contained very few free amino groups. After hydrolysis with 6N HCl for 3 hr, paper chromatography showed the presence of several distinct ninhydrin-positive spots. Several of these were provisionally identified by comparison with known amino acids run adjacently. The whitish area was still present but the "tail" had disappeared. Arginine, aspartic acid, glutamic acid, valine and leucine were now identifiable.

This 3 hr hydrolysate was evaporated to dryness, dissolved in water and carefully neutralized by the addition of solid $Ba(OH)_2$, considerably more being required than expected, probably due to the presence of free phosphoric acid appearing during the initial preparation and remaining after the HCl had been removed. When the solution was just alkaline it was filtered and the filtrate evaporated to dryness under reduced pressure. Meanwhile the residue was dissolved in dilute HCl and extracted with methyl ethyl ketone. This extract was also taken to dryness under reduced pressure. Paper chromatography was again used to study the two fractions. The filtrate, containing barium salts of the free amino acids, exhibited the expected series of amino acid spots and a trace of the white area attributed to citrate. The fraction isolated from the residue showed a single ninhydrin-positive spot identified as aspartic acid (the barium salt of which is relatively insoluble) and the typical whitish "citrate zone", with very little ninhydrin-positive surround. (Analysis showed that two thirds of the nitrogen appeared in the filtrate and one third in the residue. Citric acid analyses at this stage were of no real value as reported below).

The residual fraction was then hydrolysed for a further 15 hr with 6N HCl. Paper chromatography of this hydrolysate then showed the presence of free arginine, tyrosine, valine and an increased amount of aspartic acid. It was thus concluded that amino acids, containing two thirds of the total nitrogen, were released from the complex after 3 hr hydrolysis and that extended hydrolysis up to 18 hr released further quantities of amino acids. The most striking features were the appearance of tyrosine only after extended hydrolysis, and the fact that the degraded peptide after 3 hr hydrolysis exhibited minimal positive reaction to ninhydrin despite its containing at least four amino acids. The results of the quantitative study indicated that release

of ninhydrin positive material must be practically complete after 18 hr hydrolysis, at least 95 per cent of the total nitrogen being released either in ninhydrin reactive form or in basic amino acids, after this time.

Ratio of citric acid to nitrogen in the complex

The precise ratio of citric acid to nitrogen has varied in a number of preparations and is obviously affected by destruction of citric acid during isolation of the complex and particularly during the preparation of hydrolysates. The method of analysis is very specific and any modification of the citric acid component will result in a low value. Ratios varying from 1·5: 1 to 5·2: 1 have been obtained. The ratio in the re-precipitated dentine minerals was 5·2: 1 and, as little destruction of citric acid would be expected at this stage, this may be of significance, though, should some of the citric acid not be associated with the complex, the true ratio may be considerably lower.

Preliminary observations on ox bone

When ox bone (tibia) was treated in precisely the same manner as the human dentine, a complex containing citric acid and peptide was obtained. Qualitative paper chromatography showed that it was very similar to that isolated from human dentine. It is intended to subject this complex to quantitative amino acid analysis and to pursue the study of its structure concurrently with the further investigations of the preparation from human dentine.

DISCUSSION

Though citric acid has been recognized as a constituent of dentine for some years (Free, 1943) and was reported earlier in bone (Dickens, 1941), its exact status in mineralized tissue has received little attention. Indeed while there have been various studies on the effect of vitamins and rachitogenic diets on the level of bone citrate (Nicolaysen and Eeg-Larsen, 1953) little has been done to increase understanding of the form in which it occurs in such tissues.

The present studies have shown that when dentine or bone is dissolved in dilute acid, citric acid goes into solution from which it is later recovered in association with a peptide. Most studies of the organic components of these tissues have been concerned with the fraction insoluble in dilute acid, while little attention has been paid to the small amount of organic material which dissolves.

However, Stack (1951) reported that during decalcification with dilute acid, nitrogenous compounds corresponding to 2·1 mg per cent of mucopolysaccharide and 7·1 mg per cent "collagen" went into solution. Our investigations have confirmed that some of the collagen does in fact go into solution. When the minerals are re-precipitated by making the solution alkaline, some of this "collagen" is adsorbed, though most is removed by washing the precipitate with water. Despite this, our final preparation did contain nearly 30 per cent of collagen as a contaminant. It is considered that this was probably adsorbed on to the re-precipitated minerals

while the citrate–peptide complex was actually linked chemically to the calcium, presumably via the free carboxyl groups of the citric acid.

In more recent studies, such as that of HUGHSTON, EARLE and BINKLEY (1959), ethylenediamine tetra-acetic acid has been used to bring about demineralization, this being followed by analysis of the insoluble collagen fraction. We have found that this method of demineralization also releases the total citric acid component together with its associated peptide. It seems clear, therefore, that in the intact tissue citric acid must be linked to the mineral phase, while it is presumably still associated with the peptide so readily isolated on freeing it from its mineral associations. It has been found, in the course of various studies of this complex, that it readily becomes linked to calcium, barium, etc., and can also form an ammonium salt. The former are practically insoluble while the latter is readily water soluble but almost insoluble in methyl ethyl ketone. When obtained from acid solution the complex is soluble in the latter reagent. It appears, therefore, that the complex contains some free carboxyl groups, though it seems likely that others must be involved in amide or peptide linkages. The carboxyl groups which are free in the isolated complex might well be linked to calcium in the intact tissue.

The amino acid analysis (Table 1, column c) indicated that the peptide hydrolysate is very rich in ammonia and arginine with moderate proportions of valine, aspartic acid, leucine and two unidentified substances (II and III). Significant quantities of phenylalanine, isoleucine and tyrosine are also present. The peptide is clearly highly basic and quite different in amino acid composition from collagen, reticulin and elastin. Arginine is the chief amino acid constituent, while the content of ammonia, assuming that it was all originally present in the peptide, is sufficient to account for not only possible amide groups modifying the side chain carboxyl groups of the aspartic and glutamic acids but for amide groups on terminal carboxyls as well. The possibility also arises that ammonia may be attached to citric acid itself. Some of the ammonia may arise artificially in the course of isolation or hydrolysis of the peptide. As already mentioned the behaviour of the intact peptide with ninhydrin suggests that it contains very few terminal amino groups, although it is not certain that such groups can be assumed to be highly reactive in peptides containing upwards of four residues.

No attempt has yet been made to identify the substances whose peaks did not coincide with those of any of the usual amino acids. Peak II is close to the position of emergence of citrulline and peak III emerges from the 100 cm column in the same region as glucosamine (MOORE and STEIN, 1951). It is clear from its behaviour on the 15 cm column, however, that the substance responsible for peak III cannot be glucosamine. The first step in the investigation of these substances will be to examine the effect of an additional period of hydrolysis, since they may be peptides with an unusually high stability to hydrochloric acid.

The precise structure of the complex must depend largely on the relative proportions of citric acid and nitrogen, which as yet has proved difficult to measure. Assuming the high value of 5:1 there must be one molecule of citric acid to every 2·7 atoms of nitrogen, at the other extreme a ratio of 1·8:1 would indicate one

molecule of citric acid to every seven atoms of nitrogen. At present it is hard to envisage whether the majority of the amino acids are linked more or less directly to citric acid, or whether they are linked together as a peptide attached at only a few points to the citric acid molecules. The first degradation studies have certainly indicated that much of the amino acid content is readily freed from the complex while the linkage between tyrosine and the complex appears strong. The peptide fragment still bound to citric acid after 3 hr hydrolysis contained only one third of the total nitrogen, and appeared to contain five amino acids.

Though it was previously considered that the peptide and citric acid might well be linked mainly via tyrosine, it has become clear that, from the high proportion of citric acid in the complex and from the way in which it breaks down, the peptide and the citric acid molecules must be linked at several points. These linkages are likely to be covalent as the complex appears stable at varying pH (HARTLES and LEAVER, 1960), and must involve amino groups in such a way that few if any are free. At the same time the ammonia content indicated that there must be a high proportion of amide carboxyl while the general properties of the complex have shown that some of the carboxyl groups are free.

These results indicate that there is present in dentine a complex which contains citric acid linked to an unusual highly basic peptide, rich in arginine and ammonia, and containing aspartic acid, valine, leucine and isoleucine as major components. It is reasonable to assume from the experimental evidence that the complex may be bound to the mineral phase in the intact tissue. There is, as yet, no evidence that the complex is associated with the major organic constituents of the tissue unless such an association was by ionic linkage broken during the initial demineralization.

It is now intended to complete the quantitative analysis of the substantially similar complex isolated from bone and then to pursue the investigations of the structure of the complexes concurrently. The final stage of the programme would be the physical and biological investigation of the intact complex.

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THE EFFECT OF TETRACYCLINE ON SKELETAL DEVELOPMENT IN THE LARVAL SAND DOLLAR (*ECHINARACHNIUS PARMA*)

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Abstract—Developing larvae of *E. parma* responded adversely to sea water to which
tetracycline was added. Low concentrations of tetracycline produced delay in develop-
ment; somewhat higher concentrations resulted in inhibition of skeletal formation;
still higher concentrations completely inhibited larval development. At concentrations
which permitted skeletal development, tetracycline became incorporated into the
growing skeleton. This tissue exhibited a very pronounced avidity for and retention
of tetracycline. Concentrations which inhibited skeletal formation were found to
exert their effect specifically at the onset of skeletal differentiation.

It has been reported (Torsten, 1956) that administration of tetracycline to mice
results in its rapid and widespread incorporation in several organs and tissues. Of
particular interest was the observation that tetracycline was incorporated into bones
and teeth. More recent studies (Milch, Rall and Tobie, 1957, 1958; Bevelander,
Nakahara and Rolle, 1959) have demonstrated that, when administered to several
species of laboratory animals, tetracycline or a fluorophore derivative of this sub-
stance was not only incorporated into growing bones, but was subsequently retained
in the bones for considerable periods of time. Our interest in skeletal formation
prompted us to explore the effects of tetracycline in the skeletal system of a divergent
organism, the echinoderm larva. Our preliminary findings constitute the body of
this report.

MATERIALS AND METHODS

Eggs and sperm were obtained from mature specimens of *Echinarachnius parma*
by the KCl injection method of Tyler (1949). Each group of embryos for a single
experiment was derived from eggs of one female and sperm of one male specimen.
Filtered sea water was used throughout the experiments. Eggs and sperm were
washed several times and were combined in a finger bowl containing 200 ml of sea
water. Approximately 100 fertilized eggs were then placed in separate compartments
of a moulded plastic ice tray, to which 10 ml of a variety of concentrations of tetra-
cycline in sea water had been added. The trays were maintained in a water bath at
a temperature of 16°C throughout the experiments. Control specimens were always
cultured in the same tray in which the experimental animals were reared.

In an effort to define the mode of action of tetracycline on the developing
echinoderm larva, several modifications were made in the culture technique. Thus,

to ascertain the timing and possible reversibility of inhibitory effects of tetracycline, groups of fertilized ova were placed in sea water containing tetracycline for periods varying from 1 hr up to approximately 22 hr, i.e. the time at which the skeletal anlage normally appears. Following this treatment the larvae were returned to normal sea water. Conversely, animals reared in normal sea water for 22 hr were then placed in sea water–tetracycline mixtures.

Inasmuch as it has been established that tetracycline has an affinity for several of the heavy metals (ALBERT and REESE, 1956), experiments were also performed in which tetracycline was added to sea water in concentrations which had first been shown to inhibit skeletal formation. Calcium, in the form of $CaCl_2$, was added to these experimental culture solutions in amounts which increased their total calcium content to 1, 2, 3, or 4 times normal. In another series of experiments, magnesium, a prominent cationic constituent of sea water, was added as the chloride salt to the experimental sea water–tetracycline mixtures, again raising the concentration of magnesium to 1, 2, 3, or 4 times normal.

The addition of tetracycline to sea water reduces the pH of this medium. Accordingly, experiments were conducted in which the pH was reduced to 7·3, i.e. the hydrogen ion concentration of the sea water–tetracycline mixture which was shown previously to produce marked inhibition of skeletal formation. The pH of the medium was modified by adding appropriate amounts of HCl, as tested with a Beckmann pH meter. Addition of this acid did not appreciably increase the volume of the water in which the larvae developed.

Examination of the cultures was made periodically with a binocular dissecting microscope. A compound microscope was used to examine the larvae for details, while for examination of the skeletal elements, polarization and phase contrast equipment was utilized. A Leitz Fluorescence Lamp CS–150 with high pressure mercury source was used with appropriate filters to assess the degree of tetracycline-induced skeletal fluorescence.

RESULTS

The pertinent morphological changes which accompany the development of the skeletal system of the echinoderm larva under laboratory conditions may be outlined briefly as follows. At the time the gut begins to invaginate, two tri-radiate skeletal spicules develop in the dorso-lateral part of the body wall in intimate association with the primary mesenchyme cells (Fig. 1). At the end of 48 hr the gut has grown across the coelom. The several parts of the spicule enlarge and, by their confluence, give rise to the skeletal rods which support the "arms" characteristic of the pluteus stage of development (Fig. 4). In our experiments a pronounced variation and inhibition of these events occurred when tetracycline was added to the sea water in which the animals normally develop.

When fertilized eggs were placed in sea water–tetracycline mixtures for 30 hr, it was observed that exposure to concentrations of 8–15 mg% resulted in a delay in cleavage, and a generalized reduction in the rate of subsequent development. Concentrations of 20–25 mg% produced a high incidence of failure of skeletal differentia-

tion (Fig. 2), while concentrations above 40 mg% resulted in arrested cleavage and complete failure of larval development.

The effect of tetracycline in concentrations permitting development is progressive. This is illustrated by Fig. 2, a specimen reared in 25 mg% for 30 hr. At this stage of development the total size of the embryo compares favourably with the control specimen shown in Fig. 1. There is, however, a less well developed gut, the mesenchyme cells show abnormal arrangement, and the tri-radiate spicules are completely lacking.

In contrast to the latter situation, specimens reared in concentrations of 15 mg% are less retarded in all respects at the end of 30 hr. During the following 20 hr of development, however, several critical events normally occur which lead to the production of the pluteus stage, i.e. differentiation of the gut, and formation of arms and skeletal rods (Fig. 4). Specimens grown for 48 hr in a tetracycline concentration of 15 mg% reveal a typical reduction in size, disturbance of body form, and only vestigial skeletal rods (Fig. 3).

It is apparent from the above that inhibition in the development of embryos exposed to 15 mg% of tetracycline for 30 hr is not unduly severe. With the initiation of gastrulation, however, modification in development becomes progressively more obvious, and results in the production of malformed embryos which shortly undergo cytolysis and die.

Tests of the reversibility of the skeletal inhibitory effect of tetracycline revealed that, at any interval preceding skeletal formation, i.e. 22 hr of development, larvae which were removed from the experimental medium (20–35 mg%), washed and returned to normal sea water, proceeded to develop in a relatively normal manner. Conversely, when animals were reared in normal sea water for 21–22 hr, and then placed in a sea water–tetracycline solution at this concentration, inhibition of skeletal differentiation occurred.

While addition of calcium increased the rate of early cell cleavage during larval development, complete protection against tetracycline effects was not achieved in experimental solutions containing as much as four times the normal content of calcium. Addition of magnesium ions in similarly high concentrations failed completely to protect against tetracycline effects.

An increase in hydrogen ion concentration did not markedly modify the development of larvae or of the skeleton. It thus appears that a reduction of the pH of sea water from 8 to 7·3 by addition of tetracycline cannot account for the observed inhibition of skeletal differentiation.

Since it has been shown that tetracyclines are incorporated into growing bones and teeth, it seemed of interest to determine whether this compound was also taken up by the skeletal elements of the sand dollar. By the simple expedient of examination in ultra-violet light following growth in a sea water–tetracycline environment, it was possible to demonstrate the presence of a fluorophore in the skeletal elements of these larvae (Fig. 3). As previously reported, the tetracycline fluorophore was identified by its characteristic bright yellow fluorescence in the ultra- or near ultra-violet range.

DISCUSSION

Previous studies have shown that tetracyclines are incorporated and retained in growing bones of several species of vertebrates. Our experiments with *E. parma*, an invertebrate, have shown that specimens reared in sea water containing tetracycline undergo several modifications in development. Concentrations of 8–15 mg % produce a delay in cleavage, and when the skeletal anlage develop under these conditions, they exhibit a fluorescence characteristic of the tetracyclines. Concentrations of 25–30 mg % effectively inhibit skeletal formation, whereas higher concentrations totally inhibit larval development. The inhibitory effect of tetracycline on skeletal formation has been shown to be reversible under appropriate experimental conditions, and to manifest itself at the time of appearance of the skeletal anlage. Experimental increase in the concentrations of the cationic constituents calcium and magnesium in the sea water–tetracycline culture medium does not provide any protection against the inhibitory activity of tetracycline. Neither does hydrogen ion concentration play a role in the experimental effects observed.

Whether tetracycline is retained exclusively in the calcified structures of this invertebrate larva has not been clearly established. We have demonstrated, however, a marked avidity of tetracycline for the developing skeleton of *E. parma*, which is composed of calcite ($CaCO_3$). This is in contrast to the mineral component of the vertebrate skeletons previously tested, which is hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). From these and other considerations it appears that tetracycline may have an unusual propensity for the calcium ion which is incorporated into skeletal systems. We propose in future studies to explore this problem in greater detail.

Finally, although our interest in this study has been concerned primarily with modifications of skeletal development, it is also apparent that the entire process of gastrulation and subsequent development is severely impaired by tetracycline treatment. This phenomenon merits further investigation.

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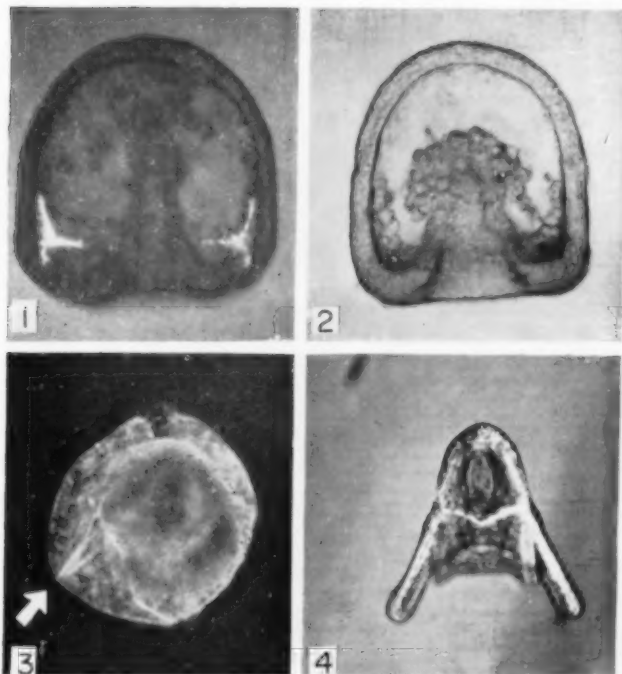
FIG. 1. Normal larva at 30 hr development. Note the well developed birefringent skeletal elements. (Polarized light). ×200.

FIG. 2. Larva at 30 hr development grown in tetracycline–sea water mixture (25 mg %). No skeletal elements are observed. (Polarized light). ×200.

FIG. 3. Larva at 48 hr development grown in tetracycline–sea water mixture (15 mg %). Note the reduction in size, modification of development, and the vestigial fluorescent skeletal element (arrow). (Ultra-violet light). ×200.

FIG. 4. Normal larva (pluteus) at 48 hr. (Polarized light). ×200.

PLATE 1

OBSERVATIONS ON THE STATUS OF COLLAGEN IN HUMAN GINGIVA

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Abstract—The amounts of total, soluble and insoluble collagens have been determined in clinically normal and chronically inflamed human gingiva, and in hyperplastic gingiva from patients receiving sodium diphenylhydantoin. The results suggest that the histological disappearance of the collagenous fibre bundles in chronic gingivitis may be due to a disorganization of the fibre bundles along with a deficient precipitation of the soluble precursors into the insoluble fibres rather than to a greatly increased rate of collagen breakdown. In hyperplasia of the gingiva, as induced by sodium diphenylhydantoin, collagen appears to be metabolized at a reduced rate, rather than being formed at an accelerated rate. On the basis of the results, suggestions are made with regard to a new approach for evaluating the influence of "endogenous predisposing factors" in chronic periodontitis.

HISTOLOGICAL investigations have established that there are three reasonably distinct systems of collagenous fibre bundles in the connective tissue of the marginal gingiva. These are held responsible for (a) the maintenance of the tone of the marginal gingiva, (b) the maintenance of a close adherence of the gingiva to the tooth, (c) the prevention of epithelial migration in an apical direction along the root surface of the tooth and (d) the unification of the gingival margin with the underlying tissues (ARNIM and HAGERMAN, 1953).

Inflammation of the gingiva is accompanied by disintegration and loss of identity of the fibre bundles, as observed in histological preparations (GOLDMAN, 1952). As a result the epithelium becomes separated from the enamel, and migrates apically on the root surface to an extent apparently determined by the presence of intact fibres in the deeper parts of the tissues. Although usually slow, gingivitis in most cases being a chronic condition, the process is progressive unless treated. Upon the removal of injurious agents (deposits, bacteria) the clinical signs of inflammation usually disappear. Subsequently, gingival fibres are rebuilt and a re-attachment to the tooth occurs. Thus it is evident that the collagen fibres of the marginal gingiva are of considerable importance for the integrity of the periodontal tissues.

So far the collagen of the gingiva has been examined by histological means only. In view of recent advances in the knowledge of the biochemistry of collagen (GUSTAVSON, 1956; JACKSON, D. S., 1957a; JACKSON, S. F., 1957; SCHMITT, GROSS and HIGHBERGER, 1955), it was thought to be of interest to study the collagen of the gingival tissues by chemical methods.

In connective tissues there are two forms of collagen, a soluble form and the insoluble collagen fibre. Fibrogenesis involves the synthesis by the fibroblast of a soluble precursor which is secreted into the ground substance. Jackson (1957) has suggested that soluble collagen may be converted into the insoluble fibre both directly by aggregation of a number of precursor particles, and indirectly by way of reticulin. In both cases the transformation of the soluble precursor into the insoluble fibre may be brought about by interaction with sulphated mucopolysaccharides (Jackson, 1953, 1954). What is included under the term "soluble collagen" depends on the solvent used. The commonly used solvents include water, neutral salt solutions, acid citrate buffer and dilute acids. Accordingly the collagenous fractions obtained upon extraction are called water soluble, neutral salt soluble or acid soluble. There are reasons to believe, however, that the various soluble fractions do not represent distinct metabolic products, but rather arbitrary fractions from a continuous series of collagens of decreasing solubilities (Green and Lowther, 1959). This is supported by the discovery that the neutral salt soluble fractions and the acid citrate soluble fractions have similar amino acid composition (Jackson, Leach and Jacobs, 1958). Furthermore the two fractions cannot be distinguished physico-chemically (Grassmann, Hannig, Endres and Riedel, 1957; Orekhovich and Shpikiter, 1957; Jackson, Leach and Jacobs, 1958; Robertson, Hiwett and Herman, 1959).

Acidic solvents will extract all of the precursors, and also reticulin. Neutral salt solutions, on the other hand, will extract the precursors of reticulin, but not reticulin itself. The water soluble fraction, obtained in a manner to be described, may represent a lower molecular weight fraction of the neutral salt soluble collagen as suggested by Jackson and Fessler (1955), or even an earlier precursor. The main point, however, in the present connection is that in connective tissues there are two major collagen fractions present, one soluble, the other insoluble, and that the bulk of the soluble collagen probably constitutes precursor material to the collagen fibres.

Furthermore it should be emphasized that under certain circumstances fibrogenesis may be reversible. That is, the insoluble form of collagen may break down via the soluble collagens. This implies that at any one time a fraction of the soluble collagen represents catabolic products. The relative amounts of soluble collagen, derived from synthesis and from physiological breakdown respectively, can only be guessed at. Since, however, the turnover of the insoluble fibre is very slow, it will be assumed that the major portion of soluble collagen under normal conditions in the connective tissue represents precursor material.

In inflammation it is assumed, on histological grounds, that the collagen fibres are broken down at an accelerated rate. It is true that fibres disappear in an inflamed area, but how far the disintegration is carried is not known. It could be, however, that the amounts of soluble collagen increase in an inflammatory area. This raises the question of how the insoluble collagen is disrupted during inflammation. The process is not yet entirely clear but there are several observations which may suggest an explanation (Ungar and Neuman, 1953; Sherry, Troll and Rosenblum, 1954; Thonard and Scherp, 1957). Thus it may not be necessary to postulate the presence

of specific collagenases to account for disintegration of collagen fibres. All that may be needed is that in inflammation the collagen fibres become slightly denatured because of changes in the milieu of the inflamed area. The denatured fibres are then disrupted by the action of the proteolytic enzymes (proteinases, cathepsins) of tissue and inflammatory cells.

Regardless of the mechanism, organized collagen fibres are observed to disappear in the inflamed gingiva, and the possibility arises that the soluble collagen may increase in amount.

The above considerations suggest the possibility of establishing abnormalities in the biochemistry of the collagen of a given tissue by analyses of the relative proportions of various collagen fractions present at any one time. Thus a low value for soluble collagen would indicate reduced synthesis. Although there is little information relating to increased amounts of soluble collagen due to increased synthesis, the studies by JACKSON (1957b) on connective tissue growth stimulated by carrageenin suggests that this assumption is reasonable. On the other hand a high value for the soluble fraction along with a low value for insoluble collagen could indicate either increased breakdown or reduced ability to precipitate the soluble precursor as insoluble fibres.

With regard to human gingiva, analyses of this kind could possibly throw some light on the "endogenous predisposing factors" of the host tissues in periodontal disease, in terms of permitting an evaluation of the ability of the host's cells to synthesize collagen. It should be emphasized that histological observations often do not permit an inference that in certain situations increased breakdown of collagen takes place. That fewer collagenous fibres than normal are present in a histological section may well be due to the inability of the tissue cells to synthesize the precursors, or to conditions in the connective tissue ground substance impeding the precipitation of the precursor as the insoluble collagen fibre. A pertinent example in this connection is the demonstration that in scorbutic tissues the histological picture of reduced amounts of collagen fibres is due to lack of synthesis of collagen rather than to increased breakdown (ROBERTSON and SCHWARTZ, 1953; ROBERTSON, HIWETT and HERMAN, 1959).

MATERIALS

A total of thirty-six tissue specimens was obtained from twenty-nine patients. Eight of these specimens from four patients were used for preliminary experiments, leaving twenty-eight specimens from twenty-five patients for the main experimental series.

All of the patients were under treatment for various dental diseases in the Department of Oral Surgery of the School of Dentistry, University of Oslo, during 1958/59. Most of these patients suffered from various periodontal conditions. Whenever a gingivectomy was performed as part of the treatment of a periodontal lesion, the tissue removed was acquired for analysis. In addition, five specimens of clinically normal tissue removed at the time of tooth extractions were obtained.

The tissues excised included the vestibular and oral aspects as well as interdental papillae, to the level of the alveolar bone margin.

The periodontal conditions of the patients were classified according to REICHBORN-KJENNERUD (1948).

Basically the periodontitis (*paradentitis marginalis*) is described according to whether the clinical picture is that of a chronic or acute inflammation. The present series of experiments included cases of *paradentitis marginalis chronica* only, except for five specimens of hyperplastic tissues derived from four patients receiving sodium diphenylhydantoin. Clinically, all of these tissues exhibited varying degrees of chronic inflammation.

The *paradentitis marginalis chronica* is further qualified by:

1. The degree of tooth mobility.
    (a) Superficialis: no increase in tooth mobility
    (b) Profunda: increased tooth mobility

2. The extension of the lesion.
    (a) Localisata: lesion confined to a limited area
    (b) Generalisata: lesion involving the gingiva of most, or all, of the teeth

3. The supposed aetiology.
    (a) Simplex: predominantly local causes
    (b) Complex: lesion cannot be explained in terms of local causative factors

4. The predominant clinical symptoms.
    (a) Exudativa: presence of oedema, pus or increased bleeding tendency
    (b) Haemorrhagica: pronounced bleeding tendency
    (c) Regressiva: retraction of the gingiva, slight inflammatory symptoms
    (d) Mixta: any combination of (a), (b) and (c).

Several of these various terms are then combined in order to give an accurate description of the condition.

METHODS

A. *Preparation of tissue samples*

(i). In a preliminary series of experiments (eight specimens from four patients) the freshly removed tissues were divided into small pieces with scissors. The pieces were immersed in cold, absolute ethanol. The ethanol was changed three times in 24 hr. Subsequently, the tissue fragments were treated with acetone, and then with ether, each for 24 hr in the cold. They were then dried for 3 hr at 110°C, and stored in a desiccator over phosphorus pentoxide.

(ii). In one series of experiments, directly upon removal the specimens were weighed (wet weight) and immersed in liquid nitrogen in a porcelain mortar. By means of a piston the tissues were crushed to a fine powder. In some cases the powder was lyophilized, and after it was completely dry, stored in an evacuated desiccator over phosphorus pentoxide.

In other cases the tissue powder was extracted directly (without drying) with various solvents.

B. *Extraction of tissues*

(i). Portions of the dried tissue powders were extracted with 0·1 M citrate buffer (pH 3·5). Five ml of the buffer were added to weighed (dry weight) samples of the material, and the suspension

was kept at 4°C for 24 hr. The tubes were shaken at frequent intervals. The suspension was then centrifuged, the supernatant was removed and kept for analysis, and the residue was re-extracted twice in the same manner. The combined supernatants (15 ml) were next lyophilized, and stored in the refrigerator until the chemical analyses were undertaken. (*Acid citrate soluble fraction.*)

The residues were treated twice, each time for 24 hr, with 0·1 N NaOH. After centrifugation the supernatants were tested to ensure that no collagen was present, and then discarded. Subsequently the residues were autoclaved twice, each time for 3 hr, with 5 ml of water and at a pressure of 15 lb/in² (CONSDEN, GLYNN and STANIER, 1953). The materials were filtered hot, with suction, through a filter paper on a Buchner funnel. The filtrate was finally lyophilized and stored in the cold. Tests for the presence of collagen in the residues were all negative. The lyophilized filtrate represents the *insoluble collagen fraction* of the tissues.

(ii). In five instances tissue powder in liquid nitrogen was poured directly into Pyrex centrifuge tubes. Five ml of water were added, and as soon as all of the ice formed had melted, the tubes were centrifuged at 2000 rev/min for 5 min. The supernatant was removed and lyophilized. (*Water soluble fraction.*)

Five ml of 0·2 M NaCl (pH 7·4) were added to the residue and further extraction was carried out for 24 hr at 4°C. The suspension was then centrifuged, the supernatant removed and stored in the cold. The extraction was repeated twice, each time with fresh 5 ml portions of the sodium chloride solution. The combined extracts (15 ml) were lyophilized. (*Neutral salt soluble fraction.*)

Five ml of acid citrate buffer (pH 3·5) were next added to the residue and the remainder of the experiment was carried out as described above, in order to obtain the acid citrate soluble and the insoluble collagen fractions.

C. *Determination of the collagen contents of the fractions*

Collagen is a protein characterized, among other features, by its hydroxyproline content (13·4%) A convenient way of determining the collagen content of tissue is to measure the amount of hydroxy-proline after hydrolysis. In the present series of experiments the total collagen content of the gingival tissue samples were measured in this manner. Furthermore the same procedure was used in establishing the amounts of collagen present in the various extracts. In all cases the samples were hydrolysed with 2 ml of 4 N HCl at 110°C for 17 hr in sealed test tubes. Following hydrolysis the hydrolysates were filtered, and then evaporated to dryness by distillation at reduced pressure at 70–80°C. The dried hydrolysates were then dissolved in suitable amounts of water, desalted when necessary, and the amounts of hydroxyproline present were determined on aliquots of the solutions by the colorimetric method of MARTIN and AXELROD (1953). On the basis of the amounts of hydroxyproline found, the amounts of collagen present in the samples were computed.

RESULTS

The preliminary series of experiments showed that collagen determinations can be made on small amounts of gingival tissues (5 mg dry weight or less), and that the amounts present are of the order of from 11 to 40 per cent of the dry weight of the tissue. The individual results however were very variable and not reproducible within reasonable limits in different pieces of the same tissue. In part this may be due to variations in the collagen content of different parts of the gingiva around any one tooth. Also the ratio of epithelium to connective tissue must be expected to vary in gingival tissue fragments according to the part of the gingiva to which they belong as well as to the clinical condition present.

Furthermore the tissue pieces had been exposed to ethanol, acetone and ether. Although these reagents may not interfere with the determinations of the total collagen content of tissues, this kind of treatment does not lend the tissues to extraction of soluble collagen.

For these reasons the results of the preliminary experiments were not considered sufficiently reliable to be tabulated here.

The deep-freezing and homogenization procedure described above enabled tissue samples to be prepared without risk of alteration by exposure to reagents. This method enables the average collagen content or the tissues to be determined in any one patient at the time of removal, but does not permit its determination in particular areas.

The results obtained are listed in the following tables.

TABLE 1. DETERMINATIONS OF THE COLLAGEN CONTENT (% OF DRY WEIGHT) OF CLINICALLY NORMAL GINGIVAL TISSUES

Specimen no.	Age in years	Sex	Acid citrate extractable collagen	Insoluble collagen	Total collagen on the basis of addition of fractions	Total collagen determined directly
142	7	F				29·7
144	21	F				27·9
145	11	F	1·7	27·3	29·0	25·1
146	13	F	1·8	30·9	32·7	34·0
147	26	F	1·5	34·3	35·8	38·5
Average			1·7	30·8	32·5	31·0

TABLE 2. THE COLLAGEN CONTENT (% OF DRY WEIGHT) OF THE GINGIVA IN PARADENTITIS MARGINALIS CHRONICA PROFUNDA

Specimen no.	Age in years	Sex	Acid citrate extractable collagen	Insoluble collagen	Total collagen on the basis of addition of fractions	Total collagen determined directly
114	57	F	0·5	23·0	23·5	24·1
116	35	F	1·5	28·5	30·1	31·0
117	35	F	1·7	20·5	22·2	23·0
121	34	M				20·2
123	26	F				25·2
124	41	F				22·8
127	31	F				24·0
128	47	M				27·0
129	58	F				24·5
137	27	F				29·6
139	47	M				11·9
143	64	F	1·3	29·3	30·6	31·6
Average			1·3	25·3	26·6	24·5

TABLE 3. THE COLLAGEN CONTENT (% OF DRY WEIGHT) OF THE GINGIVA IN VARIOUS TYPES OF PARADENTITIS MARGINALIS CHRONICA

Specimen no.	Acid citrate extractable collagen	Insoluble collagen	Total collagen on the basis of addition of fractions	Total collagen determined directly
(a) *Profunda regressiva*				
123				25·2
127				24·0
128				27·0
137				29·6
139				11·9
143	1·3	29·3	30·6	31·6
Average				24·9
(b) *Profunda exudativa*				
114	0·5	23·0	23·5	24·1
121				20·2
124				22·8
Average				22·4
(c) *Profunda exudativa et regressiva (mixta)*				
116	1·5	28·5	30·0	31·0
117	1·7	20·5	22·2	23·0
Average	1·6	24·5	26·1	27·0
(d) *Superficialis**				
115	1·1	16·1	17·2	20·1
118	0·9	12·3	13·2	15·6
Average	1·0	14·2	15·2	17·9

* Specimen no. 115 was from a 29 yr-old female, specimen no. 118 from a 27 yr-old male.

TABLE 4. THE COLLAGEN CONTENT (% OF DRY WEIGHT) OF HYPERPLASTIC
GINGIVAE FROM PATIENTS RECEIVING SODIUM DIPHENYLHYDANTOIN

Specimen no.	Age in years	Sex	Acid citrate extractable collagen	Insoluble collagen	Total collagen on the basis of addition of fractions	Total collagen determined directly
113	32	M	1·3	30·1	31·4	32·8
132	33	F				29·6
134	9	F				26·3
136	35	M				31·0
Average						29·9

TABLE 5. DETERMINATIONS OF COLLAGEN (% OF WET WEIGHT) IN FRACTIONS
EXTRACTED DIRECTLY FROM FROZEN, POWDERED GINGIVAE

Specimen no.	Age in years	Sex	Wet weight of tissue (g)	Water soluble collagen	Neutral salt soluble collagen	Acid citrate soluble collagen	Total soluble collagen	Insoluble collagen	Total collagen
120	47	F	0·393	—	0·04	0·23	0·27	2·57	2·84
125	38	M	0·184	0·04	0·05	0·20	0·29	2·90	3·19
126	29	F	0·855	0·04	0·06	0·14	0·24	7·00	7·24
130	37	M	0·312	0·02	0·07	0·12	0·21	4·30	4·51
131	35	M	0·149	0·05	0·05	0·10	0·20	2·86	3·06

DISCUSSION

A. *Materials and methods*

Before considering the implications of the results, certain points with regard to the methods and the materials must be discussed.

The method employed appeared reliable for the determination of the average amounts of collagen present in the tissues. Whenever sufficient material was available, two or more tests were carried out on different samples of the same tissue preparation. The variations in the measurements of the collagen present averaged ±0·7 per cent of the dry weight. Furthermore, as seen from the tables, the total collagen values as determined directly, were not very different from the values obtained by adding the various collagen fractions, in spite of the number of manipulations involved in the extraction procedures.

It is uncertain whether or not the methods employed were optimal for accurate estimation of the various soluble collagens of the tissues. Thus it may be that the extractions should have been prolonged, although preliminary experiments, as well as the experience of other investigators, suggested that extractions carried out in the manner described are fairly exhaustive and representative.

The hydrolysis procedure employed in the present series of experiments was arrived at after a number of preliminary tests involving variations in the normality of the acid, the time of hydrolysis and the treatment of the hydrolysate prior to the hydroxyproline determinations. By the method used, the results obtained were reproducible. In any case, with regard to the determinations of the collagen contents of the tissues, they were carried out in an identical manner in all cases, so the results are regarded as representative when it comes to comparing the relative amounts of various collagens present in the different tissues.

As was pointed out previously, tissues derived from different areas of the mouth of the same patient were analysed in three cases (115/131, 126/136, 128/139). From Table 2 it is seen that in the case 128/139, the results are entirely different (27 per cent and 11·9 per cent of collagen, respectively). However, specimen 139 was anomalous, presenting a single value very different from the other results. It is scarcely explained on the basis of the derivation of the tissue, because the clinical diagnosis was the same in both cases. In the other two cases the measurements were in fairly good agreement. By assuming a water content in human gingiva of 80 per cent, the results obtained with the wet tissue (Table 5; 131) correspond to 1·0 per cent of soluble and 14·3 per cent of insoluble collagen on a dry weight basis. As seen from Table 3 (d, 115) the corresponding results of the analyses of the dry tissue were 1·1 per cent and 16·1 per cent. In the case of specimens 126 and 136, the corresponding results are 36·2 per cent and 31·0 per cent. It should be added that the water content of the gingiva was not precisely determined in these experiments. The tissues which were dried were weighed before and after the removal of the water, and an average water content of 80 per cent was found. The procedure, however, was not entirely standardized. Furthermore, it is likely that the water content of normal gingiva will differ somewhat from that of the inflamed tissue.

As described above, the hydroxyproline content of all of the various collagens was assumed to be the same. This seems to be true for the acid citrate and neutral salt varieties, as well as for insoluble collagen, but it is not established for the water soluble fraction. An effort was made to determine this in the water extracts of the present series of experiments by precipitating this collagen in a pure form in the manner described by GREEN and LOWTHER (1959). Because of the very small amounts of material available, however, the results were not conclusive.

As is evident from the tables, the number of tissues included in the various clinical categories (Table 3) is not entirely sufficient to permit adequate comparison. In part the reason for this was that the chemical analyses were carried out in ignorance of the clinical diagnosis. Also, of course, the decision to perform a gingivectomy in each individual case was made from the point of view of the patient's welfare. On the other hand the results listed in Table 3 are to an extent supplemented by those in Table 5. The results therefore are regarded as suggestive and will be discussed in terms of the present knowledge of collagen, although they should be accepted as preliminary only, particularly since the patients were not of the same age.

B. *Implications of the findings*

In comparing the information compiled in Tables 1 and 2, the data on soluble collagen show no appreciable differences in the amounts present in clinically normal and chronically inflamed tissues, except in one case (Table 2; 114).

Since there was no unusual accumulation of soluble collagen in these cases of chronic inflammation, it appears that the insoluble collagen is not broken down to the soluble stage to a significant degree. Furthermore the data suggest that even in the chronically inflamed tissues the soluble precursors continue to be formed by the cells. As is to be expected, there were individual variations, depending on the degree of inflammation present (Table 3) and also, to some extent, on the age of the patient.

With regard to the insoluble collagen, there was, on the average, less present in the chronically inflamed tissues than in the clinically normal tissues. The difference, however, was not as pronounced as was expected on the basis of histological observations, except in one case (Table 2; 139). In individual chronically inflamed tissues the amounts of insoluble collagen present were sometimes (Table 2;116, 143) even higher than in individual clinically normal cases (Table 1; 144, 145), although they did not approach the highest values found in the healthy tissues (Table 1; 146, 147). These findings suggest that insoluble collagen is also formed in chronic inflammation and furthermore that the "breakdown" of insoluble collagen is not greatly accelerated. Again there are individual variations, but taken together the data on both soluble and insoluble collagen suggest that less collagen is synthesized in tissues where the inflammatory symptoms dominate (Table 3, b and d) than when the clinical picture is that of a regressive (degenerative) process (Table 3, a and c).

Thus it would seem that the histological interpretations of the changes in chronic periodontitis are somewhat misleading. The findings here presented indicate, first of all, that the disappearance of the collagenous fibre bundles as observed in the light microscope is primarily the result of disorganization of the fibre bundles, and secondarily due to a greatly enhanced disappearance of collagen, or a greatly reduced ability of the tissue cells to form the precursors. This would also explain the rapid re-appearance of the gingival fibre bundles upon healing of the lesion. Secondly these experiments suggest that in chronic inflammation, collagen is not broken down to the soluble stage to any great extent or permanently. That is, the breakdown of collagen in chronic inflammation may not occur by simple reversal of the process of fibre formation. Thirdly, the information obtained emphasizes the dynamic situation in the tissues, that chronic periodontitis is not a one way process only, but that synthesis of tissue components also goes on—at times to an appreciable degree. Thus the disease process appears to be one where at times tissue disorganization predominates, whereas in other periods there is a significant amount of synthesis of tissue elements. This confirms earlier observations (SCHULTZ-HAUDT, 1957) to the extent that the main difference between clinically healthy and chronically inflamed gingiva is one of degree rather than of type.

One objection to the interpretations of the findings is that the unit volume of the tissues taken up by cells (and exudate) per sample has not been considered. The results therefore may not be applicable to restricted local areas of the tissue surrounding

one tooth. On the other hand the fair amount of tissue analysed in each test, as well as the parallel tests included, suggest that the results are representative as averages.

Some of the results here presented also suggest the importance of considering endogenous predisposing factors in periodontitis. It is believed that the status of collagen in the gingiva may contribute to a better evaluation of these than has hitherto been possible.

Since it appears well established (WAERHAUG, 1952; SCHULTZ-HAUDT, 1957) that bacteria contribute to the development of periodontitis, it is important that the resistance mechanisms of the host tissues operate in a normal way. Apart from the specific host resistance mechanisms against bacteria, such as antibody formation and phagocytosis, resistance depends upon the maintenance of normal tissue metabolism. Any interference with the normal physiology and biochemistry of the cells and tissues may reflect itself in a specific or non-specific decrease in host resistance. Examples of interfering conditions are inadequate nutrition and hormonal disturbances.

One indicator of normal cell metabolism is that the tissue cells synthesize extracellular components at a normal rate. Collagen is a connective tissue component, and abnormalities in the synthesis of this protein might suggest disturbances in the tissue metabolism which in turn may modify tissue resistance. In other words, it might be expected that the histories of the patients where the analyses of the gingivae showed definitely reduced values for both soluble and insoluble collagen (114, 115, 118, 121, 130) would reveal general disturbances as well—these representing the "endogenous predisposing factor". In three cases this was so (114, 121, 130); in the remaining two cases there was no past history of any disturbance pertinent to this discussion. This might only mean that the records are incomplete, or that there may be reasons to subject the patients to examination of, for example, the diet or the hormonal status.

In spite of the limitations of the material, the findings suggest that endogenous predisposing factors should be considered in chronic periodontitis. They further indicate that by refinement of the type of technique used in the present experiments the relative importance of these may be evaluated in a quantitative manner. This could lead to the development of tests which could be helpful in deciding the kind of treatment to be instituted in particular cases, as well as with regard to the prognosis of any one case.

The data on gingival hyperplasia induced by sodium diphenylhydantoin will not be discussed in detail here, since this study is being continued and extended. From Tables 4 and 5 (126) it is seen however that the total collagen content of these tissues was comparatively high, although not uniformly higher than in clinically normal tissues as would have been expected on the basis of histological observations. The reason for this may have been that the ratio of epithelium to connective tissue may have been widely different from normal cases, and also that all of these tissues were affected by varying degrees of inflammation. Neither were the total amounts of soluble collagen present unusually high in the cases where this was measured. Tentatively it is proposed that collagen is not formed at an accelerated rate in these cases, but rather that the physiological breakdown of the collagenous fibres is slowed down.

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THE EFFECT OF BILIRUBIN ON TOOTH DEVELOPMENT IN THE RAT

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Abstract—The production of hyperbilirubinaemia in the rat has been attempted in
four ways: (a) the injection of haemolytic antiserum, (b) ligation of the bile duct,
(c) injection of a bilirubin solution and (d) the injection of bilirubin solution 2 days
after ligation of the bile duct. Method (d) was found most successful in producing a
marked and sustained rise in the serum bilirubin. A histological study was made
of the effect of such hyperbilirubinaemia on the continuously growing rat incisor, and
it was found that a bilirubin level of 30 mg/100 ml was associated with green staining
of dentine and with enamel hypoplasia.

CLINICAL studies (MILLER and FORRESTER, 1959) have shown the association of
kernicterus and of jaundice with enamel hypoplasia and with intrinsic green staining
of dentine. The object of the present study was to produce in the rat a jaundice
simulating as closely as possible the haemolytic jaundice of the newborn in man,
and to observe the effect of such a jaundice on tooth formation.

HSIA, ALLEN, DIAMOND and ELLIS (1953) reported that human kernicterus was
usually associated with a level of serum bilirubin not less than 20 mg and usually
over 30 mg per 100 ml, of which half gave the indirect reaction to the Van den Bergh
test. In order to study whether the dental changes associated with kernicterus were
due to bilirubin, similar levels should be produced in an animal.

Preliminary studies to produce jaundice in rats were made by the following methods:

1. Haemolysis was produced by the injection of antiserum, according to the method of BESSIS
   and FREIXA (1947). No detectable level of bilirubin could be produced by this method on
   account of the efficiency of the rat liver in excreting bilirubin. This study, which was made
   on fifty-nine rats of varying ages, included a series on two groups of pregnant rats, as an
   enamel hypoplasia had been observed in one pregnant rat following an injection of the
   antiserum.
2. Injection of bilirubin solution. The effect of the injection of bilirubin solution was studied
   on twenty-seven rats. It appeared that the injection of 2·5 ml bilirubin solution produced
   a plasma level of 4·6 mg bilirubin/100 ml plasma at the termination of injection. This level
   dropped to 1·3 mg bilirubin after 2 hr and was completely eliminated at 24 hr.
3. At the eighth day following bile duct ligation a level of 7·8 mg bilirubin/100 ml plasma was
   reached, of which 80 per cent was direct-reacting bilirubin.

As neither the injection of bilirubin nor the ligation of the bile duct alone produced
a jaundice approaching a level of 30 mg bilirubin/100 ml, it was decided to combine
the two methods in an attempt to raise the bilirubin level of the rat to a level similar
to that in haemolytic disease of the newborn.

The work of EDLUND (1948) has shown that after ligation of the bile duct secretion
of bile continues, with consequent distension of the duct. This was confirmed in the

preliminary studies of the present studies. It was thus necessary to permit a period of time to elapse after ligation of the bile duct before the injection of bilirubin so that some degree of distension of the duct and some degree of hyperbilirubinaemia might occur; thus the injected bilirubin might be able to raise the level of bilirubin in the blood. If the bilirubin were injected at the time of the ligation it is possible that the whole of it might be excreted into the bile duct without raising the level in the blood stream.

METHODS

Bile duct ligation

Under ether anaesthesia, the abdomen was opened at the mid-line, the bile duct was located and divided between two cotton sutures tied 2 mm apart in the middle third of the duct. The muscle layer was closed with continuous gut sutures and the skin with interrupted cotton thread sutures. The whole operation was usually completed in 15–20 min.

Injection of bilirubin

The bilirubin was obtained from a commercial source and dissolved according to Weinbren's method (personal communication) as follows: 10 mg of bilirubin was added to 5 ml of a solution containing 0.5% Na_2CO_3 and 0.52% NaCl. The bilirubin solution was shaken automatically for 5 min and any residue centrifuged down. When tested as serum, it was found that 6.35 mg of bilirubin was dissolved in 5 ml of the solution. Thus, even in this alkaline solution the full amount of 10 mg bilirubin did not dissolve. The possibility of incorporating the bilirubin with albumen was considered but the high viscosity of the solution made it unsuitable for injection through a narrow needle.

White albino rats of the Wistar strain were used and the majority were bred in the department. They were kept in wire mesh cages and fed on a commercially prepared standard diet.

Under ether anaesthesia, the bilirubin solution was injected into the femoral vein at a rate of 1 ml per 6 min by an injection apparatus.

Estimation of bilirubin

All blood samples were collected by exsanguination from the aorta under ether anaesthesia, and to allow for the effects of the cycle of bilirubin secretion (EDLUND, 1948) the collections were always made between 10.00 and 12.00 hours. Usually 5–7 ml were obtained for testing. Each collecting tube had been treated with 10 units of herapin to prevent clotting. The serum was tested for bilirubin according to the method of MALLOY and EVELYN (1937).

Histological technique

On killing the rat, the head was removed from the body. The skin was removed and the upper and lower jaws were separated, the upper jaw being split between the upper incisors. The teeth and their supporting bone were placed in an ample volume of 10% formol saline.

After at least 48 hr fixation, decalcification was carried out in 10% nitric acid and paraffin-embedded sections 7 μ thick were prepared. Ground sections were prepared with a slitting wheel and rubbed down as necessary, using an Aloxite stone (Carborundum 201 A).

EXPERIMENTAL PLAN

In Series 1 a further study was made of the level of serum bilirubin at intervals up to 10 days after ligation of the bile duct. The preliminary studies had indicated the possibility of sex differences; this was also studied.

In Series 2 a study was made of the serum bilirubin levels following a combination of the methods of bile duct ligation and the injection of bilirubin solution.

Series 3: following Series 1 and 2 a histological study was made of the teeth of rats following hyperbilirubinaemia produced by Series 2 method.

Series 1

Tables 1 and 2 show the levels of bilirubin found in rats following bile duct ligation alone. The male rats (Table 1) had a higher bilirubin level at the second day than the female rat (Table 2); by the tenth day the level in the females was higher than that in the males. The tables also show the percentage of the total bilirubin that was direct-reacting and it appeared that throughout the 10 days 70–80% of the bilirubin was in the direct-reacting form. From the preliminary studies and from the work of EDLUND (1948) it was expected that the level of bilirubin would gradually increase with time after bile duct ligation. The variations in this series are possibly due to individual rat or litter variations. The figures are therefore interpreted only generally. There does not appear to be great variation in the level of bilirubin between the second and the eighth day.

TABLE 1.   SERUM BILIRUBIN LEVELS IN MALE RATS (200 g) AFTER
BILE DUCT LIGATION

Time between bile duct ligation and death (days)	Bilirubin (mg/100 ml serum)		% bilirubin giving direct reaction
	Total	Direct	
2	7·05	6·1	86
	9·2	8·35	
	9·23	7·45	
6	8·56	6·13	73
	8·65	6·55	
10	6·75	5·27	79
	5·9	4·65	

TABLE 2. LEVEL OF SERUM BILIRUBIN IN FEMALE RATS (200 g) AFTER
BILE DUCT LIGATION

Time between bile duct ligation and death (days)	Bilirubin (mg/100 ml serum)		% bilirubin giving direct reaction
	Total	Direct	
2	6·34	3·71	67
	6·47	4·9	
4	6·82	5·55	81
	6·5	5·35	
	6·37	5·15	
5	9·5	6·5	
6	5·2	4·35	85
	5·45	4·72	
	6·62	5·45	
8	6·87	5·0	80
	7·7	6·58	
10	9·65	6·3	65
	10·4	6·84	
	11·2	7·85	
	12·1	8·35	

Series 2

In this series bilirubin solution was injected into rats on the second day after bile duct ligation. Table 3 shows that following an injection of 2 ml of the solution into 150 g rats a level of approximately 20 mg bilirubin/100 ml was achieved, of which 50 per cent was of the direct-reacting form at the completion of the injection. The percentage of direct-reacting bilirubin rose to 70 per cent in 3–4 hr and was the same after 24 hr. Table 4 shows the result of injections of 3 ml and 5 ml of the bilirubin solution. The injection of 3 ml of bilirubin solution produced, at the termination of the injection, an average level of 17 mg bilirubin, approximately half of which was in the direct-reacting form. As the injection of 3 ml of bilirubin did not appear to raise the level of the bilirubin greatly above that reached with 2 ml injections, tests on the 3–4 hr level and 24 hr level were not pursued. The injection of 5 mg bilirubin solution produced an average level of 30 mg at the termination of the injection which fell to 17 mg by 18 hr; the proportion giving a direct reaction rising from 50 per cent at 0 hr to 70 per cent at 18 hr. It appeared that the injection of 5 ml of the bilirubin solution could produce transiently a serum level of approximately 30 mg/100 ml of which half was in the direct-reacting form at the time of the injection.

TABLE 3. EFFECT ON SERUM BILIRUBIN OF INJECTION OF 2 ml OF BILIRUBIN SOLUTION 2 DAYS AFTER BILE DUCT LIGATION (150 g MALE RATS)

Time from injection (hr)	Bilirubin (mg/100 ml serum)		% bilirubin giving direct reaction
	Total	Direct	
0	24·4	11·78	51
	17·7	9·87	
3–4	16·5	11·4	71
	20·4	15·3	
24	17·0	15·6	71
	*21·7		

* 200 g female rat received 2·4 ml.

TABLE 4. EFFECT ON SERUM BILIRUBIN OF INJECTION OF BILIRUBIN SOLUTION 2 DAYS AFTER BILE DUCT LIGATION (200 g MALE RATS)

Time from injection to death (hr)	Volume of bilirubin solution injected (ml)				% bilirubin giving direct reaction
	3		5		
	Bilirubin (mg/100 ml serum)		Bilirubin (mg/100 ml serum)		
	Total	Direct	Total	Direct	
0	20·8	11·05	33·7	17·5	52
	14·5	7·7	31·0	16·6	53
			27·9	11·7	43
18			15·4	11·6	75
			16·5	10·7	65
			19·7	13·4	69

Series 3

In this series the bile duct was tied and the rats were injected with 5 ml bilirubin 2 days later, as in Series 2. The rats were killed on the sixth day after the injection.

Control rats had the bile duct tied and received either no injection or injection of the Na_2CO_3–NaCl solution without bilirubin.

The injection of 5 ml of the bilirubin solution produced death in many rats; consequently in a further series 3 ml was used. The injection of 5 ml of the control solution proved more difficult than the injection of the 5 ml of bilirubin solution; in several rats, following the injection of the control solution, it was difficult to arrest the haemorrhage from the injection site in the femoral vein. In many of these rats subsequent haemorrhage caused death.

The final bilirubin levels at the sixth day following the injection are shown in Table 5. It will be seen that the ultimate levels are little different in Series 3 from those in Series 1 at similar time levels. It is interesting that two control rats had higher levels of bilirubin than the rats injected with bilirubin (Table 6).

TABLE 5. SERUM BILIRUBIN LEVELS AT SIXTH DAY AFTER INJECTION
(200 g MALE RATS)

Rat no.	Volume of bilirubin solution injection (ml)	Bilirubin (mg/100 ml serum)		% bilirubin giving direct reaction
		Total	Direct	
1	3	6·7	4·65	70
2		8·45	5·52	65
3		9·0	6·65	74
4	5	7·52	5·35	71
5		7·62	5·82	76

TABLE 6. MALE RATS (200 g): INJECTION OF 5 ml OF Na_2CO_3–NaCl SOLUTION

Time from injection to death (days)	Bilirubin (mg/100 ml serum)		% bilirubin giving direct reaction
	Total	Direct	
6	11·0	9·7	88
2	9·2	6·62	71
*2	4·65	3·1	67

* moribund.

Controls
FEMALE RATS (175 g): INJECTION OF 3 ml OF Na_2CO_3–NaCl SOLUTION

Time from injection to death (days)	Bilirubin (mg/100 ml serum)		% bilirubin giving direct reaction
	Total	Direct	
6	7·07	5·3	75
6	5·6	4·22	76
Bile duct ligation but no injection	11·15	8·5	74

HISTOLOGICAL FINDINGS
Preliminary studies

In one of the preliminary experiments following the injection of haemolytic anti-serum into a 200 g female rat a line of interrupted enamel formation was seen in the lower incisor (Fig. 1). On post-mortem examination this rat appeared jaundiced, was found to have been pregnant, and also had a neoplastic or parasitic growth in the liver. A blood specimen was not obtained from this rat as it died overnight. In further studies using injection of haemolytic anti-serum into pregnant rats other cases of interrupted enamel formation were not produced.

In the series, following bile duct ligation without added injection the dentine of the incisor teeth showed vascular inclusions when the bilirubin level had been 10 mg/100 ml or more at death.

Series 3. *Bile duct ligation followed by injection of bilirubin*

On the decalcified sections of the incisor teeth there was a line of interrupted formation in the enamel matrix. This line was present in those rats which received bilirubin and also in the control rats which received the control solution. There was also a similar line in the enamel matrix of the rat which received bile duct ligation but no injection. These lines appeared to be the same. There was a certain amount of interglobular dentine in all sections. In one rat which received 3 ml of the bilirubin solution there was a notch in the enamel matrix filled with an amorphous mass of haematoxylin staining material (Fig. 2). This appeared to have originated from ameloblasts which had degenerated in this region. Between the notch and the line of interruption there was a small amount of enamel matrix which indicated that the upset which produced the notch occurred approximately 2 days after the upset which produced the line.

In the ground sections of the incisor teeth no marked alteration of the enamel and dentine was observed with transmitted light. There was a faint band of green-stained dentine in the incisor teeth of the three rats which received 5 ml of bilirubin solution. There was no staining of the dentine in the teeth of those rats which received 3 ml of bilirubin solution nor was there any staining in the teeth of the control rats. The dentine in the region of the green stain showed interglobular areas.

Using polarized light the area of the green stain stood out markedly from the dentine on either side of it. This demonstrated that the green stained area had altered optical properties which could indicate disturbed calcification. It was not possible to obtain satisfactory photographs of the green staining.

DISCUSSION

Throughout the series the main problem was to produce and maintain a deep jaundice. The efficiency of the liver of the rat in the conversion of indirect to direct reacting bilirubin and the rapid elimination of bilirubin from the blood stream was the main difficulty.

In the preliminary series employing haemolytic antiserum enamel hypoplasia occurred in the teeth of one rat which died after becoming markedly jaundiced, and was found on post-mortem examination to have a growth in the liver and also to be pregnant. Normal and pregnant rats were injected with haemolytic anti-serum, but in no other rat was a jaundice observed nor was bilirubin detected in the serum. It was apparent, therefore, that some factor other than the injections of haemolytic anti-serum or the pregnancy was operating in this one rat to produce the jaundice; this factor may have been connected with the growth in the liver.

In the studies with bile duct ligation alone the dentine showed vascular inclusions at a bilirubin level of 10 mg/100 ml serum. The bilirubin in the blood was mainly of the direct-reacting type in this series.

In Series 3, employing bile duct ligation and injection of bilirubin, a notch occurred in the enamel matrix of one rat and green staining was produced in the dentine of the other rats. In this series a level of 50 per cent indirect-reacting bilirubin was maintained for a brief period; the level fell to 25 per cent indirect-reacting bilirubin by 3–4 hr. It appeared that the presence of a high level of indirect-reacting bilirubin was associated with the occurrence of dental hypoplasia. High levels of direct-reacting bilirubin, though producing green staining of the dentine, did not appear to have the same effect on dental development. Green staining of human dentine has been observed in a case of congenital atresia of the bile duct without dental hypoplasia; it appears therefore that the green staining may result from the direct-reacting form of bilirubin. It is difficult to understand why injection of 3 ml of solution produced an enamel fault whereas in other rats the 5 ml injection produced no enamel lesion. The same pattern has been seen in the human where a high level of bilirubin is not always associated with an enamel lesion and quite moderate levels may precede an enamel fault. This suggests that other factors, possibly the different hepatic efficiency in different individuals, play complementary parts in the production of enamel faults.

The injection of greater quantities of bilirubin might have produced further lesions but the difficulty of studying the effect of control injections of Na_2CO_3 in NaCl solution prevented the further investigation of the method. Until it is possible to produce a high level of indirect-reacting bilirubin in the blood of a rodent with a continuously growing incisor it will not be possible to examine this possibility.

The distribution and calcification of the Haversian systems of the rat differ more markedly from the human pattern than do those of the rabbit, so that research on bone growth is being pursued on the rabbit (VAUGHAN, personal communication). It may well be that the physiology of the teeth of the rat differs from the physiology of the human teeth to a similar extent. It thus appears that it will be necessary to examine the enamel and dentine of continuously growing incisors to ascertain which is the most suitable animal on which to continue studies of dental formation.

It is concluded that these experimental findings add further evidence to the clinical findings that hyperbilirubinaemia may affect dental development and may be the aetiological factor in the dental malformation associated with kernicterus.

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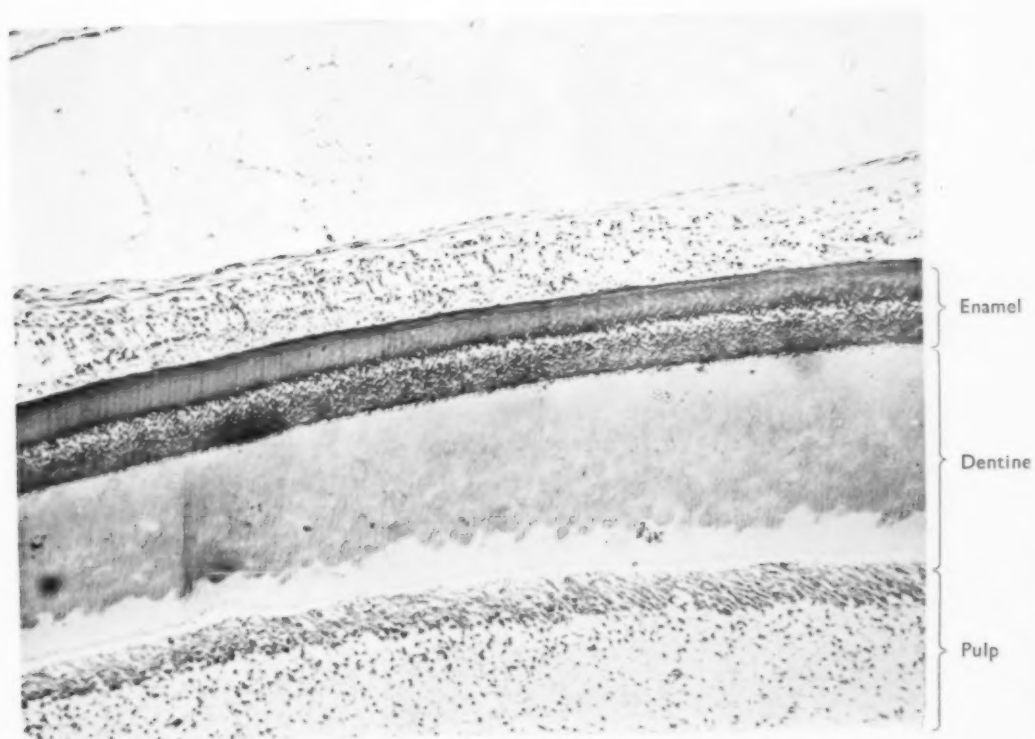
FIG. 1. Interrupted enamel formation associated with the injection of haemolytic antiserum. Haematoxylin and eosin. ×85.

PLATE 1

FIG. 2. Disturbance in enamel matrix formation following injection of bilirubin and bile duct ligation. Haematoxylin and eosin. ×360.

PLATE 2

DENTAL CARIES AND COMPOSITION OF BONES AND TEETH OF WHITE RATS: EFFECT OF DIETARY MINERAL SUPPLEMENTS

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Abstract—The ash, calcium, phosphorus, carbon dioxide and magnesium in the teeth of white rats and in certain bones, were studied in relation to a drastically altered calcium, phosphorus and magnesium content of the diet and in relation to the dental caries experience. The dietary mineral variations affected the composition of certain of the calcified tissues, particularly those which were being calcified during the experimental period. Chemical changes in the teeth and bones, however, did not relate to the dental caries experience. There was no evidence that the $CO_2:PO_4$ ratio of dental tissues was a factor in the dental caries experience.

A striking and consistent reduction in dental caries occurred as a result of adding Na_2HPO_4 to a basal cariogenic skim milk powder diet. The cariostatic effect of $CaCO_3$ was equivocal. There was no reduction in caries when $MgCO_3$ or $Mg_3(PO_4)_2$ were dietary supplements.

THE material of this report is concerned with the effect of pronounced changes in dietary calcium, phosphorus and magnesium, on experimental dental caries in white rats. The caries results were studied in relation to the ash, calcium, phosphorus, carbon dioxide and magnesium content of bones and teeth. In addition, the experiments have added substantially to our previous evidence concerning the cariostatic effect of mineral phosphates—particularly dibasic sodium phosphate. Previous literature on the effects of phosphates on experimental caries was reviewed in our previous publications (McCLURE, 1958, 1959; McCLURE and MULLER, 1959).

Prior to this publication SOBEL and HANOK (1948, 1958) reported that diets of low-calcium high-phosphorus and high-calcium low-phosphorus content produced respectively, high $PO_4:CO_3$, low $Ca:PO_4$ ratios; and low $PO_4:CO_3$, high $Ca:PO_4$ ratios in the tibia and femur and in the dentine and enamel of incisor and molar teeth of the cotton rat. These ratios in turn related directly to similar ratios in blood serum. The interesting results, however, were not consistent in the cotton rat (SOBEL and HANOK, 1958) when compared with the Wistar rat (SOBEL and HANOK, 1948). SOBEL (1955) also reported that a high $CO_3:PO_4$ ratio in dentine and enamel was conducive to dental caries susceptibility. On the other hand, WYNN, HALDI, BENTLEY and LAW (1956, 1957) and McCLURE (1958) found no chemical components of the dentine and enamel, i.e. ash, calcium and phosphorus (McCLURE, 1958) and ash, calcium, phosphorus, magnesium and carbon dioxide (WYNN *et al.*, 1956, 1957) which could be correlated with the dental caries experience.

The general plan of the experiments is indicated in the tabulation of the data (Tables 1–3). Two series of experiments, designated as Studies I and II, are described. The compositions and analyses of the diets are shown in Table 1. The dental caries data are shown in Tables 2 and 3. The analytical chemical results are presented graphically in Figs. 1–4.

STUDY I

There were four series of experiments in this study, i.e. 1a–c, 2a–c, 3a–c and 4a–c (Table 2). The diets were the same in all four series of experiments, i.e. diets a, b and c (Table 1). In these experiments we compared the effects of diets containing high and low calcium: phosphorus ratios. These ratios were as follows:(a), 1·2 (control), (b) 4·2 (test) and (c) 0·3 (test) (Table 1). The mineral supplements were $CaCO_3$, 2·77% and Na_2HPO_4 5·41% (Table 1).

TABLE 1.   DIET COMPONENTS AND ANALYSIS (PER CENT)

Study	I			II				
Diet	a*	b	c	5a	5b	5c	5d	5e
Skim milk powder†	35·00	35·00	35·00	35·00	35·00	35·00	35·00	35·00
Cerelose	18·00	18·00	18·00	18·00	18·00	18·00	18·00	18·00
Corn starch	45·00	42·23	39·59	45·00	43·37	41·99	42·70	41·32
Liver powder	2·00	2·00	2·00	2·00	2·00	2·00	2·00	2·00
$CaCO_3$	—	2·77	—	—	—	1·38	—	1·38
Na_2HPO_4	—	—	5·41	—	—	—	—	—
$MgCO_3$	—	—	—	—	1·63	1·63	—	—
$Mg_3(PO_4)_2$	—	—	—	—	—	—	2·30	2·30
Ash	3·21	5·42	7·85	2·97	3·77	5·02	4·75	5·56
Ca	0·50	1·71	0·50	0·47	0·47	1·23	0·47	1·00
P	0·42	0·41	1·50	0·38	0·40	0·40	0·79	0·77
Mg	0·04	0·04	0·04	0·05	0·46	0·47	0·53	0·50
Ca/P	1·19	4·17	0·30	1·24	1·18	3·07	0·59	1·30

* Representative analysis of diets used in Study I (Table 2).

† Autoclaved (see McClure, 1958).

A vitamin concentrate was given orally once a week to all rats to provide approximately 1500 units of vitamin A, 105 units of vitamin D and 5 mg of vitamin E.

For these experiments, with the exception of 1a–c (Table 2), the rats (N.I.H. Sprague Dawley strain) were housed two per cage. In Experiments 1a–c ten rats were housed initially in each large cage. Mortality reduced this number so that during the first 50 experimental days the number of rats per cage on diets a, b and c averaged 7·5, 5·0 and 5·0 respectively. Two weeks later, at the termination of the experiment, these averages were 6·0, 4·5 and 4·0 respectively. Only rats surviving 50 days were studied. In this series (1a–c) and in the other three series of this study, control and test groups contained litter-mated rats among which there were 20–30 litters represented. The rats were started at weanling age (21 days) weighing 30–35 g.

TABLE 2. DENTAL CARIES EXPERIENCE IN RATS FED DIETS VARIED IN THE Ca:P RATIO BY ADDITION OF $CaCO_3$ AND Na_2HPO_4 SUPPLEMENTS

Experiment	1a	1b	1c	2a	2b	2c	3a	3b	3c	4a	4b	4c
Diet	a	b	c	a	b	c	a	b	c	a	b	c
Number of rats	28	28	19	38	28	30	36	33	36	28	27	27
Average daily gain* (g)	0·6	0·6	0·5	1·1	0·8	0·5	1·1	0·7	0·5	2·2	1·1	1·2
Rats with caries (per cent)	100·0	82·1	78·9	94·7	100·0	36·6	100·0	78·7	36·1	92·9	80·8	37·0
Carious lower molars (per rat)	5·0	3·4	1·9	3·2	4·0	0·5	4·1	2·2	0·6	2·5	3·1	0·8
Standard error	±0·3	±0·2	±0·4	±0·3	±0·3	±0·2	±0·3	±0·3	±0·1	±0·2	±0·4	±0·2
Caries severity score (per rat)	22·8	12·0	4·3	8·6	13·1	1·1	10·8	4·2	0·9	6·6	9·7	1·5
Standard error	±3·0	±2·1	±0·9	±1·2	±1·9	±0·3	±1·4	±0·6	±0·2	±0·8	±1·7	±0·5

* The initial weight of these rats was 30-32 g and they were on experiment approximately 63 days.

After a period of 56–63 days on the various diets the rats were killed and the femurs separated and cleaned after mild autoclaving to facilitate the removal of soft tissue. The heads were similarly processed by autoclaving (approximately 10 min at 15 lb) and the teeth examined for caries as previously described (McClure and Muller, 1959). The teeth, mandibles and maxillae were then isolated for chemical analyses.

Teeth and bones were pooled by experimental groups, dried, ground to pass a 60-mesh sieve, fat extracted with alcohol and ether, and dried at 105°C. Enamel and dentine of the molar and incisor teeth were separated by the flotation method of Crowell, Hodge and Line (1934).

Phosphorus was determined by the differential spectrophotometric method of Gee and Dietz (1953) employing the molybdivanadophosphate complex. Calcium was determined by a semi-micro method involving the precipitation of calcium as the oxalate, oxidation of the oxalate with an excess of standard ceric sulphate, and back titration of the excess with ferrous ammonium sulphate solution. This method was an adaptation of the ultramicro method of Kirk (1950). Magnesium was determined by the colorimetric method of McCann (1959). Carbon dioxide was determined in the Warburg apparatus by the method of Deakins and Burt (1944). When sufficient material was available, determinations of calcium and magnesium were also made by standard gravimetric procedures involving a double precipitation of calcium as the oxalate with final weighing as CaO, and double precipitation of magnesium as the ammonium phosphate with final weighing as $Mg_2P_2O_7$ (Hillebrand, Lundell, Bright and Hoffman, 1953).

Dental caries results

A detailed description of the dental caries which is most characteristic of this autoclaved skim milk powder diet was reported previously (McClure, 1958; McClure and Folk, 1953). In general the typical and most prevalent carious lesions occur on buccal surfaces of lower molar teeth. The dental caries data which apply to the lower molar teeth (Table 2) show that the unsupplemented basal diet "a" pro-produced a 92·9–100 per cent caries incidence, averages of 2·5–5·0 carious lower teeth per rat and severity scores varying from 6·6 to 22·8. As previously observed (McClure and Folk, 1955) multiple-housing apparently promoted the development of caries (Experiments 1a–c).

The most significant cariostatic effect in these four series of experiments was due to the mineral supplement, Na_2HPO_4. This is evident in the consistent data for Experiments 1c, 2c, 3c and 4c (as compared with their controls), which show a lower caries incidence and in particular a marked reduction in the number of carious teeth and in the caries severity score. These consistent results due to Na_2HPO_4 agree with our previous evidence of a striking inhibition of caries by Na_2HPO_4 (McClure and Muller, 1959; McClure, 1959) when added to wheat cereal diets.

The caries results associated with a $CaCO_3$ supplement after four experiments were variable and the data remain equivocal. It appears that when very severe caries was induced by the cariogenic basal diet alone, particularly in Experiment 1a, the

$CaCO_3$ supplement was cariostatic (1b). In Experiment 3b, as compared with 3a, some caries inhibition resulted, due apparently to the $CaCO_3$ supplement. However, in Experiments 2a (compared with 2b) and 4a (compared with 4b), the $CaCO_3$ exerted no such effect.

STUDY II

This study was concerned with the effect of dietary magnesium supplements. Thus in Experiments 5a and 5c, a low content of magnesium (0·05 %) was compared with high dietary magnesium (0·46 %) while the calcium: phosphorus ratio approximated 1·2. In Experiments 5b–e, magnesium remained essentially constant whereas the calcium: phosphorus ratio was varied, i.e. 1·18 (5b), 3·07 (5c), 0·59 (5d) and 1·30 (5e) (Table 1). The experiments and analyses of bones and teeth in this study were conducted in a manner similar to the first study.

The dental caries results of this study are presented in Table 3. The rats in all these five groups developed a high incidence of caries, 90·7–100 per cent. As compared with the control diet (5a), the diets containing $MgCO_3$ (5b), $MgCO_3$ plus $CaCO_3$ (5c) and $Mg_3(PO_4)_2$ plus $CaCO_3$ (5e) resulted in a considerable increase in caries severity scores over the controls (5a). This occurred in three diets with variable calcium: phosphorus ratios.

With $Mg_3(PO_4)_2$ as the only mineral supplement (5d), there was no apparent effect on the dental caries experience. In general these results with the magnesium supplements reflect the somewhat uncertain situation which pertains to $CaCO_3$ (Table 2). In four experimental groups, however, an increased magnesium content of the diet had no caries inhibitory effect.

Weight gain

Tables 2 and 3 also show the rats' average daily gain in weight. Differences in weight gain may be attributed to food consumption and to the kind of dietary mineral supplement. It should also be noted that a high grade skim milk powder diet was used in Experiments 4a–c and 5a–c. This milk powder, although autoclaved, apparently provided more available lysine and therefore better weight gains (McCLURE and FOLK, 1953) than did the lower grade milk powder which was used in the other experiments. Gain in weight was not associated with the dental caries experience.

BONE AND TOOTH ANALYSES—RELATED TO DIETARY MINERALS AND DENTAL CARIES EXPERIENCE

The results of the chemical analyses of bones and teeth pooled for each individual experiment have been combined and are presented graphically in Figs. 1–4. Fig. 1 shows for the first study the ash content and the calcium, phosphorus, calcium: phosphorus ratio, carbon dioxide and magnesium in the ash of enamel, dentine and bone—classified in relation to the calcium: phosphorus ratio of the diet. As evident in Fig. 1, there was a tendency towards increased ash, particularly in upper incisor

dentine, and in first and second molar dentine and third molar dentine—the result of the mineral-supplemented diets. The ash of lower incisor dentine apparently was not increased.
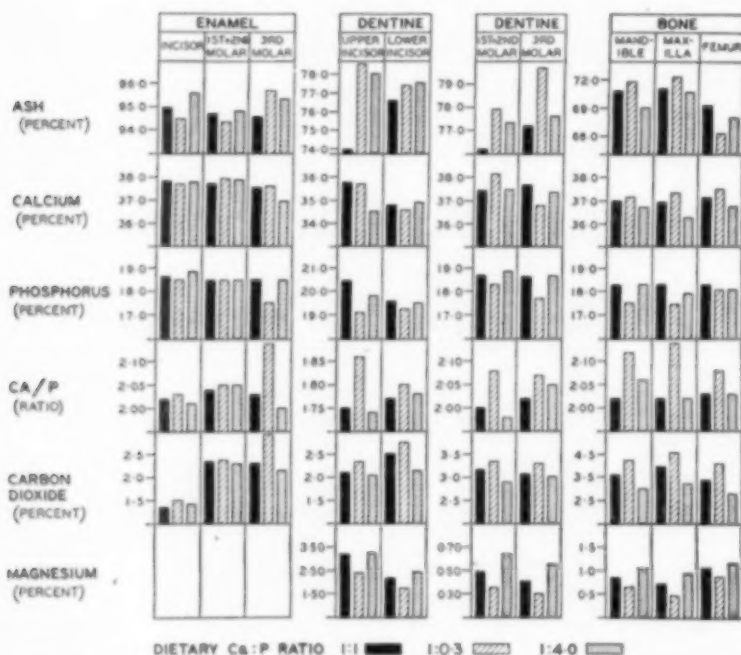
Fig. 1. The ash content and the calcium, phosphorus, calcium:phosphorus ratio, carbon dioxide and magnesium in the ash of enamel, dentine and bone in relation to the dietary calcium:phosphorus ratio.

The high calcium diet "b" increased the calcium:phosphate ratio in the ash of the third molar enamel, upper incisor dentine, first and second molar dentine, and also in the ash of mandibles, maxillae and femurs. This effect was not apparent in ash of lower incisor dentine.

Carbon dioxide was increased somewhat in the ash of the third molar enamel and in the ash of bones, due to the high calcium diet "b". High phosphorus diet "c" apparently decreased (although only slightly) the carbon dioxide in the bone ash and in the ash of lower incisor dentine. There were other variations in the carbon dioxide content of the tooth ash but these were not consistently related to the dietary mineral supplements. The carbon dioxide of the incisor enamel ash was consistently lower than molar, enamel, dentine and bone ash.

Figs. 2–4 summarize all the group-analytical data obtained on the enamel of the incisors (Fig. 2), lower molars (Fig. 3) and third molars (Fig. 4). The data are plotted in relation to the average dental caries scores. Included in these three figures are

results of the analyses of teeth obtained from a control and test group of rats which are part of another study. The test rats in this case received a supplement of 1·5 per cent calcium acetate. The calcium, phosphorus, magnesium, and Ca:P ratio of the control and test diets were respectively 0·03%, 0·36%, 0·14% and 0·08 (control), compared with 0·41%, 0·34%, 0·14% and 1·21 (test).
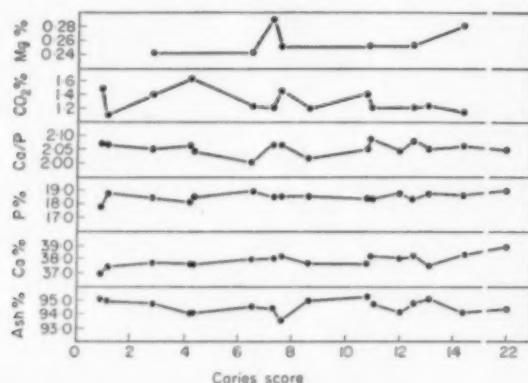
FIG. 2. Analysis of incisor enamel of groups of rats, related to the average dental caries score of the group.
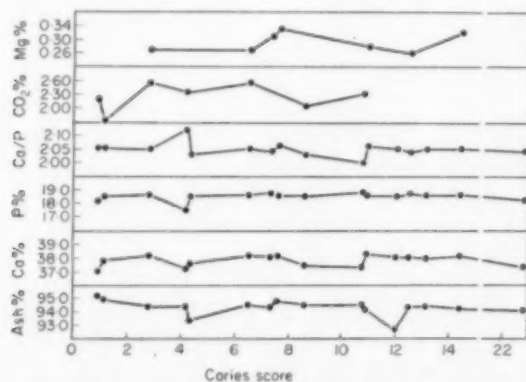
FIG. 3. Analysis of lower molar enamel of groups of rats related to the average dental caries score of the group.

Although not appearing in these three figures the progressive increase in caries scores as shown, was accompanied by an increase in the caries incidence. Thus in those groups in which the average caries scores were less than 2, 2·0–4·3, and 6·0 or above, the percentage of rats with caries was respectively 36·0, 78·0, and 90·0–100·0. Each point on these graphs represents an individual experiment and the data pertain to 19–30 rats in each experimental group. As noted above, the bone and tooth

specimens were pooled for analysis by experimental groups. Some 150 specimens representing approximately 570 rats were analysed for ash, calcium, phosphorus, carbon dioxide and magnesium.
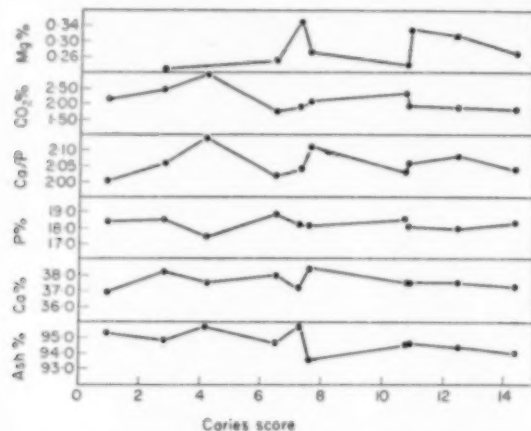
Fig. 4. Analysis of the third molar enamel of groups of rats related to the average caries score of the group.

None of the analytical results, as is apparent in the graphs, afforded any evidence that variations in the minerals as analysed in these teeth or bones, were related to the dental caries experience.

Effects of magnesium

On inspecting our results with respect to the effect of magnesium supplements in the diet (Study II), it became apparent that there was a considerable increase in magnesium and a corresponding slight decrease in the Ca:P ratio in the bones, and to a lesser extent in the dentine. Thus the control femur ash, Experiment 5a, con-

Table 3. Dental caries experience in rats fed diets varied in Ca, P and Mg content by $Ca(C_3H_5O_3)_2 \cdot H_2O$, $MgCO_3$, $CaCO_3$ and $Mg_3(PO_4)_2$ supplements

Diet*	Mineral supplement in diet	Number of rats	Daily gain† (g)	Rats with caries (%)	Carious teeth (no. per rat)	Severity score (ave. per rat)
5a	Control	30	2·2	90·7	3·4±0·3	7·6±0·9
5b	$MgCO_3$ 1·63%	30	2·1	100·0	3·7±0·3	10·9±1·6
5c	$MgCO_3$ 1·63%, $CaCO_3$ 1·38%	29	1·8	100·0	4·0±0·3	14·4±2·3
5d	$Mg_3(PO_4)_2$ 2·30%	30	2·3	100·0	2·9±0·3	7·3±1·1
5e	$Mg_3(PO_4)_2$ 2·30%, $CaCO_3$ 1·38%	30	2·2	100·0	4·2±0·3	12·5±1·8

* See Table 1.
† The average initial weight of the 5a–e rats was 50·4 g, and they were on experiment 56 days.

tained 38·11 per cent Ca, 18·54 per cent P, 0·78 per cent Mg, Ca/P 2·06; whereas femur ash from 5b contained 37·15 per cent Ca, 18·74 per cent P, 1·31 per cent Mg and the Ca : P ratio was 1·98.

These results indicate an apparent substitution of magnesium for calcium, which is of interest because of the possible association of magnesium with bone crystal structure. Thus in the light of our previous observations a considerable percentage of magnesium in bone can be leached with water or dilute acid, without affecting its calcium content appreciably (McCann and Bullock, 1955). This would support the probability that magnesium had substituted for calcium on the surface of the apatite crystal.

The CO_2 content appeared to be affected only slightly, if at all, by this increase in dietary magnesium. No relationship between dental caries and a variation in the magnesium composition of the teeth was apparent in the results of this study.

Table 4. Pigmentation of incisor teeth and iron content of the enamel

Diet	5a	5b	5c	5d	5e
Pigment score*	2·62 ± 0·12	1·20 ± 0·10	0·38 ± 0·09	1·98 ± 0·11	1·47 ± 0·11
Fe %	0·19	0·14	0·07	0·18	0·13

* See Larson (1958).

A noticeable effect of the high magnesium diets (particularly in association with a high Ca low P content of the diet) was a decrease in incisor pigmentation and in the iron content of the enamel (Table 4). It may be noted in this connection that it has been reported (Pindborg, 1953) that a lack of incisor pigmentation occurs in rats given diets deficient in Ca and P as well as magnesium. A different condition, however, was apparent in our rats, i.e. the lowest level of pigmentation (Experiment 5c) was associated with lowest femur ash, indicating a lack of available phosphorus.

DISCUSSION

The calcium and phosphorus mineral supplements as fed at the levels used in these experiments, influenced the mineral composition of the ash of these rats' bones and teeth. This was evident in those tissues, particularly bones, which were under-growing growth and calcification during the course of the experiments. In this regard it may be noted the rats' molar dentition presents an obviously disconcerting situation for this type of study. At the time of initiating these dietary experiments (at weaning age), the first and second molars were practically fully erupted and calcified, and the third molars completed their calcification early in the experimental period. The molar teeth of the rat increase in size, however, and in newly formed dentine, as the animal grows. In the incisor teeth, the amount of new tissue formed during the experimental period almost equalled the amount of tissue originally

present. Growth and developmental factors therefore may account for the fact that the enamel of the first and second molars remained practically unaffected by the mineral supplements, whereas the third molar enamel underwent some change, particularly in the Ca:P ratio and carbon dioxide content. Similarly an inspection of the results for molar dentine suggests that the dentine in all the molars may have been influenced chemically by the added dietary minerals. Somewhat contrary to expectation the enamel of the incisors remained quite constant for all the rats regardless of dietary minerals.

With regard to our evidence that the caries experience was not related to tooth composition, it is noteworthy that the dental caries which is characteristic of this particular cariogenic diet, is most severe and most prevalent on the lower third molars. The first and second molars generally have a lower caries incidence and less severe caries. It is of interest, therefore, that although the third molars did show some changes chemically, these teeth consistently developed the highest incidence of caries, irrespective of their composition. Similarly with the presence of Na_2HPO_4 (Experiment 3) no caries developed in the first and second molars, whereas in the third molars, whose chemical composition was changed, there was no such dramatic reduction in caries.

Under the dietary conditions used, the suggestion that a higher $CO_2:PO_4$ ratio in the teeth would tend to increase caries susceptibility gains little support. The findings shown in Figs. 2–4 do not indicate any such relation. In addition it may be noted that the individual group-chemical analyses for the experiments in Study I (Table 2), indicated no change in the $CO_2:PO_4$ ratio of the first and second molar enamel and a small but significant increase in the $CO_2:PO_4$ ratio in the third molar enamel. However, the caries severity scores both increased and decreased in comparison with their controls, in these several experiments. In addition an inspection of our data for the test and control groups of rats given calcium acetate shows that there was an apparent decrease in dental caries in the test group, but this was associated with a considerable increase rather than decrease in the $CO_2:PO_4$ ratio of the molar teeth. (Molar ratio×100 increased from 6·54 to 9·15).

It may also be worth considering that an explanation of caries inhibition based on a supposition that the carbonate of enamel is preferentially dissolved by acid may be open to question. Although such a solubility effect may be true for bone, Hendricks and Hill (1950) have shown that this is not true for enamel. More recently Hills and Sullivan (1958) have obtained evidence that there is no preferential solution of carbonate from human enamel in citrate and lactate buffers at pH 4–7. The presence of CO_2 in the solutions also had no effect on the solubility of the enamel.

In conclusion it may be emphasized that dibasic sodium phosphate proved to be a consistent and significantly cariostatic agent when added, in this case, to a cariogenic skim milk powder diet. This anticaries action was not related to changes in ash, calcium, phosphorus, carbon dioxide and magnesium content of the enamel or dentine. In contrast to this significant anticaries result with dibasic sodium phosphate, calcium carbonate, magnesium carbonate, and magnesium phosphate as dietary mineral supplements, gave equivocal anticaries effects.

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METACHROMATIC REACTIVITY OF MAMMALIAN SUBMAXILLARY GLANDS

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Abstract—Submaxillary gland metachromatic activity was studied in six different mammals. The results obtained demonstrate that although certain rodent glands (rats and rabbits) contain cytological components which resemble mucous cells, these cells do not show metachromatic activity. In higher mammals (dogs, cows, hogs and humans) the submaxillary gland always exhibited metachromasia. No relationship could be detected between the metachromatic activity of cells and their hydrolytic enzymatic content. These findings underline once more the need for further studies on the structure and function of the submaxillary glands in mammals.

INTRODUCTION

PREVIOUS investigations (QUINTARELLI and CHAUNCEY, 1959; CHAUNCEY and QUINTARELLI, 1959a,b) concerned with histochemical localization of hydrolytic enzymes in the major salivary glands of mammals and humans have indicated that the "activity patterns" of the various species studied differ both quantitatively and qualitatively. The greatest variation occurred among the components of the sub-maxillary gland. This was principally due to certain basic histological differences among the glands studied. Dog, cow, hog and human glands contain both serous and mucous secretory components. On the other hand rat and rabbit glands, in addition to their normal serous acini, contain "special" serous cells which morphologically resemble mucous cells but are devoid of mucin.

Past studies (BENSLEY, 1908; STORMONT, 1932) on the structure and function of the various component cells in rat and rabbit salivary glands have yielded contradictory interpretations. A desire to obtain additional information which might be of value in resolving these disputes led us to consider augmenting the histochemical studies with an investigation on the comparative metachromatic activity of mammalian salivary glands.

METHODS AND MATERIALS

Rat, rabbit, dog, cow and hog salivary glands were removed immediately after death (desanguination). Human glands were usually obtained within one or two hours after clinical death. All glands were washed with distilled water (to remove gross blood), cut into blocks 5 mm thick and representative specimens placed into Spuler and Rossman's fixative. Experimental tissues were fixed for three days,

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during which time the fixative was changed daily. Specimens were then embedded in paraffin and sectioned at 5μ. The staining solution used was safranine O, 0·1% aqueous solution. Weigert's acid iron haematoxylin (nuclei) and 1·5% aqueous fast green (cytoplasm and collagen fibres) were employed as counterstains. Additional sections were stained by the Mallory azan method or with Masson's trichrome mixture.

Since a variability in results noted in prior investigations on the metachromatic reaction of tissues (BENSLEY, 1934; SYLVÉN, 1941, 1945) was due in part to differences in technique—fixation time, section thickness, alcohol dehydration and staining time in the present study were rigidly standardized in order to minimize technical errors and permit an accurate comparison of tissues from the different animals.

RESULTS

Rat. The serozymogenic cells showed an orthochromatic reaction, visualized as a violet colour distributed homogeneously throughout the cytoplasm. The nuclei, well delineated at the periphery of the acini, were stained black. Certain of the "special" serous cells contained small purple granules in the cytoplasm. In contrast, other cells were agranular and stained only pale azure green (Fig. 1).

Rabbit. In this animal the cytoplasm of the serozymogenic cells was coloured bluish-green. Dye disposition was in the form of small filaments, producing a delicate network. The cytoplasm of the "special" serous cells was filled with a cloudy greenish material. Duct cells were green, except for a moderate orthochromatic reaction in the area above the nucleus normally occupied by the Golgi apparatus (Fig. 2).

Dog. The submaxillary gland of the dog exhibited a striking metachromatic reaction. The large mucous acini stained a brilliant orange-pink. The intracellular metachromatic material was dispersed throughout as clusters of large globules. Demilunes also stained, but their colour (deep purple) was considerably darker than the reaction presented by the mucous cells. Under high magnification it was noted that a fine green filament divided the demilunes from the mucous acini. Connective tissues and ducts were stained a bright green (Fig. 3).

Hog. Porcine gland showed definite metachromasia. The mucous acini, large and tumid, was stained pink purple. Under high magnification the contents appeared as small round globules intersected by a filamentous network irregularly distributed throughout the cytoplasm. The acinar cells were filled by this intensely staining purple material, except for a thick basal membrane which stained green. Ducts were green and contained in their lumina a pink material which may have been a secretory product (Fig. 4).

Cow. The submaxillary gland of the cow appeared as a typical mixed gland. The mucous portion was clearly metachromatic, containing pink globules which were intersected by a faint purple network. The individual mucous cells were separated from each other by interseptal fibres which stained blue. The demilunes, stained blue-violet, contained minute granules, while ducts and connective tissue were unreactive and took up the green counterstain (Fig. 5).

Human. The tetra stain in this gland showed an orange-pink metachromatic reaction in the mucous acini. Demilunes, small and flattened against the base of the mucous acini, and serous cells were orthochromatic-violet (Fig. 6).

DISCUSSION

Metachromatic reactivity. The present investigation has shown that using standard technical procedures the submaxillary glands of mammals, except for rodents, contain metachromatically staining components (Table 1). This reaction ranged from a light pink to a pink-purple, according to the species studied, and the reactive material appeared in different forms. In humans it was visualized as a delicate compact mass of intertwined filaments which entirely filled the cells, while in dogs this material appeared in the form of large globules scattered throughout the mucous cell cytoplasm. In hogs and cows the positive components were seen as clusters of small globules intersected by thin fibrillar material.

Contrary to the observation that the submaxillary gland of rabbit contains cells which histologically resemble mucous cells, it has been shown by HEIDENHEIM (1868) that the secretion from this gland does not contain mucin. With the technique employed in the present investigations no metachromasia could be detected in any of these cells. Reactivity of rat "mucous acini" was negligible. These results confirm the recent work of JACOBY and LEESON (1959), who were unable to find metachromasia in the submaxillary gland of the rat.

Although the safranine O metachromatic staining method was found to be reproducible and dependable there is still some question as to why certain cells stain in this manner and exactly which constituents are responsible for the staining reaction. According to HEMPELMANN (1940) epithelial mucin, mucous glands, ground substance of tendon, sclera, Warton's jelly, intercellular matrix of cartilage and certain other structures stain metachromatically. The observation that metachromatic staining is specific for certain tissues is supported by the work of KRAMER and WINDRUN (1954), who found that even prolonged alcohol dehydration was incapable of reversing the metachromasia of tissues known to contain sulphate esters of polysaccharides.

The results obtained in the present study show that mammalian salivary gland cells which contain mucin are capable of staining metachromatically with safranine O, while cells found in rabbit salivary glands which appear similar morphologically, but apparently are devoid of mucin, do not stain with these same agents.

Metachromasia and hydrolytic enzyme activity. The localization of hydrolytic enzymes in mammalian submaxillary glands has been previously described. However, it appears of interest to reiterate these prior observations in relation to the staining characteristics of the various species employed (Table 1). In the rat the acid phosphatase and β-D-galactosidase were not demonstrable in the "special" serous cells. On the other hand, these cells exhibited strong esterase activity. The basal membrane was heavily stained, while the cytoplasm reacted to a lesser degree. In the rabbit the distribution of all three enzymes was homogeneous to such an extent that it was not possible to discriminate between the serous acini and the "special" serous cells. In dogs the mucous cells were negative for all three enzymes. Mucous

acini in the cow contained neither acid phosphatase nor β-D-galactosidase, while hog gland demonstrated no acid phosphatase, but was weakly reactive for β-D-galactosidase. Mucous cells of both animals demonstrated esterase activity. The reaction was moderate in the cow and intense in the hog. In humans the only enzyme reaction detected in the mucous acini was a weak β-D-galactosidase activity, which was confined to the basal membrane of these cells.

TABLE. 1.   METACHROMASIA AND ENZYME ACTIVITY OF MUCOUS ACINI AND "SPECIAL" SEROUS CELLS IN THE MAMMALIAN SUBMAXILLARY GLAND

Species	Metachromasia	Acid phosphatase	Nonspecific esterase	β-D-galactosidase
Rat	±	0	4	0
Rabbit	0	2	2-5	1-5
Dog	+	0	0	0
Hog	+	0	4	1-5*
Cow	+	0	2	0
Human	+	0	0	1*

Stain reaction	Enzyme reactivity	
+ = Metachromatic	4	Intense
— = Orthochromatic	3–3·5	Marked
0 = Negative	2–2·5	Moderate
	1–1·5	Weak
	0·5	Occasional
	0	Negative

* Activity limited to basal membrane.

A vast variety of work on the functional activity of rodent submaxillary glands has been conducted in the past seventy years, but certain important questions remain unanswered. Although there is general agreement that the "special" serous or "mucoid" cells do not show metachromatic reactivity, no matter what procedure is employed, these cells are PAS positive (LEBLOND, 1950). Most investigators are in agreement with ORREL (1904) that the submaxillary glands of mice and rats are strictly serous. Furthermore, investigation of the basophilic reactivity of the "special" serous cells (NOBACK and MONTAGNA, 1947) has shown that these cells are only weakly reactive, indicating a low nucleoprotein content (CASPERSSON, LANDSTRÖM and AQUILONIOUS, 1941).

The results of the present study, when integrated with our previous histochemical findings, serve to emphasize the fact that cytological structures which often appear similar when conventional histological stains are used, actually have quite different biochemical identities. Perhaps the combined application of biochemical and morphological techniques will permit resolution of the functional significance of rodent "special" serous cells.

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FIG. 1. Rat. Safranine O, iron haematoxylin and fast green. Serous cells orthochromatic. "Special" serous cells are filled with green granules, but in some a pale violet colour is detectable. ×200.

FIG. 2. Rabbit. No metachromatic reaction. Both types of acini stain homogenously. ×200.

FIG. 3. Dog. Intense metachromatic reaction in mucous acini. Connective tissue and ducts green. ×200.

FIG. 4. Hog. Pink-purple metachromatic colour in the mucous acini. Connective tissue and ducts green. ×200.

FIG. 5. Cow. Orthochromatic reaction in demilunes. Mucous acini react metachromatically. ×200.

FIG. 6. Humans. Tetrachromatic stain represented by the metachromatic orange colour in mucous cells, deep purple stain of serious acini, black nuclei, and green ducts and connective tissue. ×200.

PLATE 1
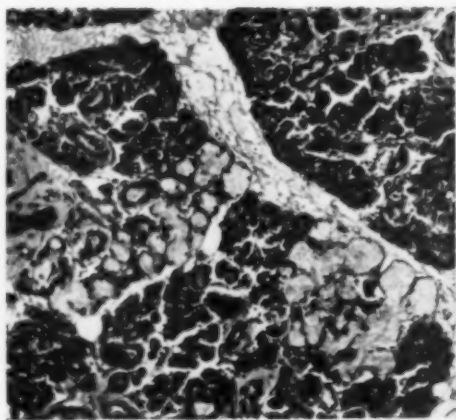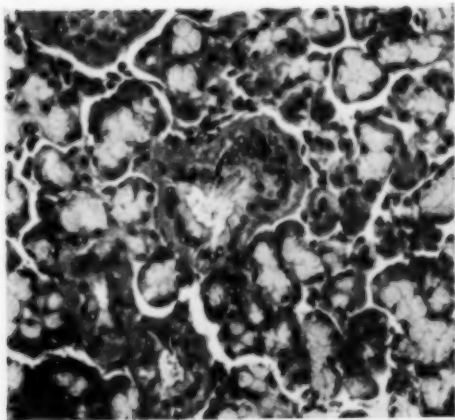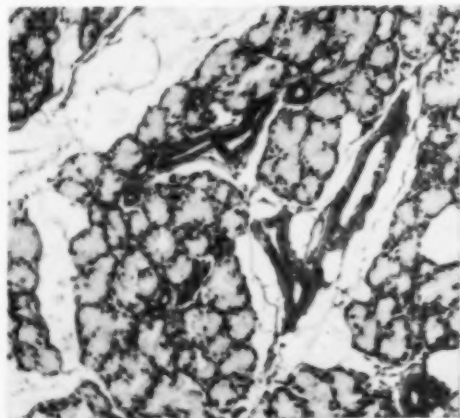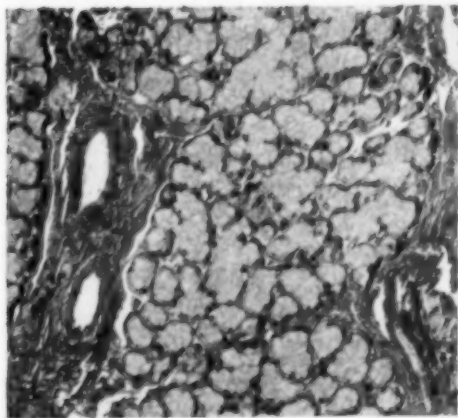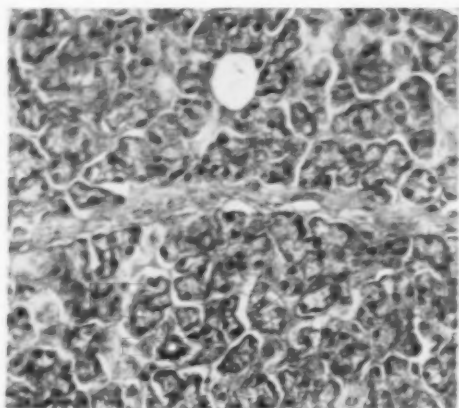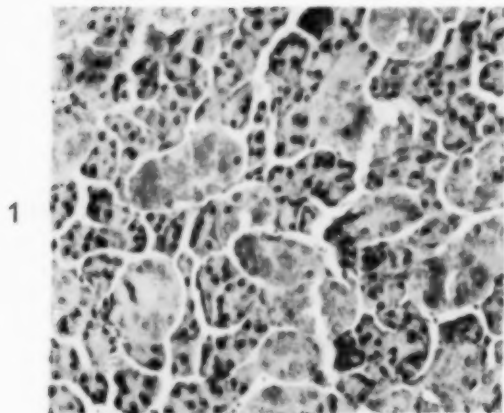
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THE VASCULAR ARCHITECTURE OF THE HUMAN DENTAL PULP

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Abstract—The technique for filling the pulpal bloodvessels of extracted human teeth with an injection mass (Kramer, 1951) has been modified and improved, and a silicone fluid has been found more suitable than methyl salicylate for clearing the injected specimens.

Using the modified technique the vascular architecture of the human dental pulp has been studied, and the main features, including the presence of direct arteriovenous connections, are described.

THE vascular architecture of a tissue or organ is usually most readily studied in preparations in which the vessels have been filled with a suitable injection mass. The material with which the vessels are filled may be chosen for direct observation, a radio-opaque material may be used and the vessel pattern studied in radiographs, or a plastic substance may be introduced into the vessels and the surrounding tissues subsequently removed. The injection mass may be perfused throughout the entire cardiovascular system (a technique usually limited to experimental animals) or the individual tissue or organs to be studied may be perfused through an accessible vessel of suitable size. The injection of the blood vessels of the dental pulp in man presents peculiar difficulties, for material suitable for injection with the teeth *in situ* is rarely available whilst the vessels at the apical foramina in extracted teeth are too small for cannulation.

Some years ago a technique was described for overcoming these difficulties (Kramer, 1951) making it possible for the pulpal vessels of extracted teeth to be filled with an injection mass. Because the method was applicable to extracted teeth, the study of human material was made easier (Russell and Kramer, 1956).

The purpose of the present communication is to describe modifications and improvements of the method described in 1951, and to report upon the vascular architecture of the human dental pulp as revealed by this method.

MATERIALS AND APPARATUS

Human deciduous and permanent teeth were used. The method of blood vessel injection was essentially similar to that described previously and depends upon the production of a pulp exposure in the crown of the tooth. When this exposure is made a few small capillary loops near the margin of the pulp are broken. When a negative pressure is applied to this exposure the contents of the vascular network are sucked out; if, during this process, the root of the tooth is immersed in Indian ink, then the

ink will be sucked in to replace the original vessel contents. Thus, the vessels are filled without the need for cannulation of the apical vessels, and without the application of any positive pressure that might rupture the walls of delicate vessels.

Two solutions are required, 3·8 % sodium citrate, and a diluted Indian ink–sodium citrate injection mass. This is prepared by diluting standard Indian ink (Windsor and Newton) to two-thirds of its original strength by the addition of distilled water, followed by the addition of sufficient crystalline sodium citrate to give a final concentration of 3·8 %. It has been found advantageous to filter this ink–citrate solution before use, as described by KELLER and COHEN (1955).

Experience has shown that better results are obtained if the vessel system is washed through with plain sodium citrate solution before the introduction of the injection mass. Therefore, the original apparatus has been redesigned to allow this preliminary washing to be carried out conveniently. Suction is supplied by a simple water pump (Fig. 1). This is connected to a water-trap flask, and then the suction line is carried
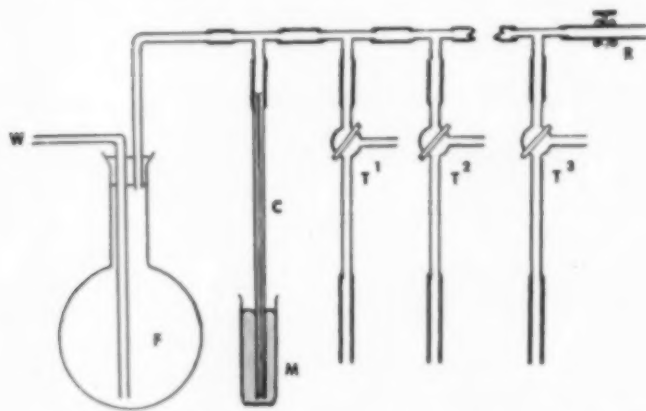
FIG. 1. Diagram of injection apparatus. W, to water pump; F, water trap flask; C, capillary tube; M, mercury reservoir; T^1, T^2, T^3, glass three-way taps; R, rubber tube with gate clamp, to provide adjustable air leak. The lower arm of each three-way tap is fitted with a short length of soft rubber tubing into which the crown of a tooth may be inserted.

to a series of glass tubing T-pieces. To the first of these is attached a capillary tube dipping into a small mercury reservoir, thus providing a simple mercury manometer for the measurement of the negative pressure applied. The number of the subsequent T-pieces depends upon the number of teeth that may have to be dealt with at any one time. To each of these is attached a three-way glass tap, the lower tube having fitted to it a length of soft rubber tubing of a size suitable to receive the crown of a tooth. It has been found convenient to have attached to some taps relatively small bore tubing to take incisor, canine and premolar teeth and deciduous molars, whilst other taps carry larger bore tubing to take permanent molar teeth. To the left-hand end of the last T-piece in the series a short length of rubber or soft plastic tubing with a gate clamp
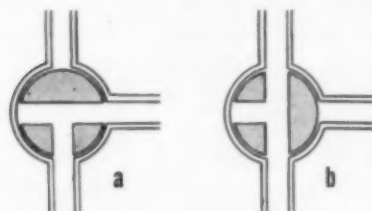
FIG. 2. The two positions of the three-way tap. In position (a) no suction is applied to the tooth, and the pulp is at atmospheric pressure. In position (b) suction is applied.

is fitted. This provides an adjustable air leak into the system for the regulation of the negative pressure. To make the apparatus ready for use, the water pump is turned on, all taps are turned to position (a) (Fig. 2) so that no air passes through them into the system, and the air leak is adjusted to give a negative pressure reading of 180 mm Hg in the capillary tube.

METHOD

Immediately the tooth is extracted it is placed in 3·8 % sodium citrate solution and taken at once to the laboratory. There, a hole is drilled well into the dentine of the crown of the tooth with a relatively large dental bur (e.g. rosehead No. 5). Through the base of this hole a pulp exposure is made with a smaller bur (e.g. rosehead No. 1). The cutting of a larger hole into the dentine initially makes it easier to see when the exposure has been made with the smaller bur. During the production of the exposure the apex must be kept moist with citrate solution, and the hole must be drilled in such a position that it will not be occluded when the tooth is fitted into the suction tube.

The crown of the tooth is now inserted into the tubing on one of the three-way taps, and soft stainless steel wire is looped and twisted round the tubing to ensure an air-tight seal between tube and tooth. A small beaker or jar of 3·8 % citrate is now placed beneath the tooth so that the entire root is immersed. When the three-way tap is turned to position (b) (Fig. 2) the suction will be applied, and the vessels are allowed to wash through for 30 min. If, at the end of this period, the citrate solution were simply removed and replaced by the injection mass, then air might be sucked into the vessels during the changing of the solution. However, if the three-way tap is returned to position (a) the suction to the tooth is discontinued and the pulp returned to atmospheric pressure without admitting air to the rest of the apparatus and therefore without interfering with the injection of other teeth being dealt with at the same time. When the beaker containing citrate solution has been replaced by one containing the injection mass, the tap is again turned to reapply the suction. Thirty minutes is allowed for the injection, the pulp is returned to atmospheric pressure, and the tooth is removed from the apparatus. After fixation in formol saline for 2–3 days the specimen is decalcified by any of the usual methods, although nitric acid is better avoided as it tends to make the dentine a darker brown than other methods. When the tooth has been decalcified the root is scraped with a small scalpel to remove the ink coating on

the cementum and periodontal membrane fragments; the cleaning of the root surface is necessary if the pulp is to be viewed clearly when the dentine has been rendered transparent. The tooth is now ready for dehydration in ascending grades of ethyl alcohol, clearing in chloroform and infiltration with the material that renders the tissues transparent. Originally methyl salicylate was used for this purpose, and it works very well. Unfortunately, this compound also has a number of disadvantages: its penetrating odour, its toxicity, its tendency to allow the specimen to absorb atmospheric moisture and become cloudy, and its remarkable properties as a solvent which make difficult the construction of suitable chambers for the manipulation and examination of the cleared teeth. It has now been found that a silicone fluid, M.S. 710 (Midland Silicones Limited, 68 Knightsbridge, London, S.W.1.) is an equally good material for rendering the tissues transparent, and it has the advantages of being odourless, non-volatile, non-toxic, water repellent and non-solvent. This latter property is a particular advantage, as Perspex chambers can be used for manipulating and photographing the cleared specimens. It has also been found that, if necessary, tissues cleared in the silicone fluid can be taken back to chloroform and subsequently embedded in paraffin wax; paraffin sections stain satisfactorily with the commonly used stains.

After transfer from chloroform to the silicone fluid, the teeth clear within a few hours. Areas of cloudiness, appearing blue/grey by transmitted light, are due to incomplete dehydration or incomplete replacement of the chloroform. The specimen should be taken back to chloroform and absolute alcohol if dehydration was incomplete.

Not all teeth show satisfactory injection by this technique; the failures are attributable to three main causes.

Firstly, it is difficult to judge the production of a small exposure in an extracted tooth, and the majority of the failed injections are due to a failure to produce the pulp exposure. Experience has shown that, whilst injection will inevitably fail in the absence of the exposure, severe damage to the pulp by deep penetration of the bur is often followed by excellent injection of the undamaged tissue. It is wise, therefore, to drill more deeply whilst making the exposure if there is any doubt about whether the pulp has been reached.

Secondly, it has been found that successful injection will be achieved only if there is a minimum of delay between extraction of the tooth and the preliminary washing through of the vessels with citrate solution. If this period exceeds 20–30 min, a failure may be expected.

Thirdly, it has been found that good injections are rarely achieved in teeth with open apices. This is probably due to a collapse of the vessels in the relatively large soft tissue mass of the apical area when the suction is applied to the coronal part of the pulp. No special difficulties are encountered in the injection of deciduous teeth with partly resorbed roots.

RESULTS

It is at once apparent that conventional histological sections give little idea of the remarkable vascularity of the dental pulp. As LEEUWENHOEK wrote in about 1682, the pulp contains "such an inconceivable number of blood vessels and other vessels as to surpass all imagination".

In the majority of teeth, the main blood vessels pass through the apical foramen or foramina, run towards the occlusal aspect of the tooth, and give rise to a rich capillary plexus near the periphery of the pulp. When the tissue is viewed through its thickest part, the vascular network is often so dense that it is difficult to see the individual vessels. However, towards the periphery of the pulp, where the thickness of the tissue is less, the finest vessels are easily seen. If these vessels are viewed at high magnification the depth of the field in focus is insufficient for the whole tooth to be examined without adjustment of focus. Therefore, if a photographic survey of large areas of the pulp under high magnification is required, a series of overlapping fields are photographed separately, after the manner of an aerial survey, and the composite picture assembled from the prints (Fig. 3). Alternatively, the whole preparation may be photographed at a lower magnification with consequently increased depth of field (Fig. 4) and the final high magnification prints produced by photographic enlargement. In addition to the better resolution obtained by the survey technique, the method has the further advantage that unwanted detail is readily thrown out of focus (Fig. 5). In multirooted teeth fine detail is also readily seen in the complex vessel networks that are often to be found connecting the vessels of the main root canals (Fig. 6).

The main arterial vessels are relatively narrow, smooth walled, and pursue a comparatively direct course towards the coronal part of the pulp. In many teeth they tend to lie towards the periphery of the pulp (Fig. 3). During their course in a mainly occlusal direction these arteries give rise to a large number of branches passing outwards to the sub-odontoblastic capillary plexus.

The veins are much larger than the arteries, their walls are more irregular, and the largest veins lie near the central part of the pulp. From the sub-odontoblastic capillary plexus the venous drainage is in the form of large numbers of vessels that mainly run obliquely inwards towards the central part of the pulp and in an apical direction. As the veins approach the apical foramen they appear to become reduced both in number and in diameter, a feature commented on by SAUNDERS (1957a) who concluded that this "suggests that the circulation in the human dental pulp is of a sluggish nature and favours the transvascular diffusion of calcium, fluorine and other ions".

Vessels connecting pulp and periodontal membrane

Whilst the arteries and veins enter and leave the pulp mainly via the apical foramina, it is not uncommon, especially in multirooted teeth, to find major vessels in other situations. Particularly in the bifurcation or trifurcation area large vessels may be found running through the radicular dentine to supply one root canal, sometimes appearing to contribute more to the root canal vascular system than the vessels entering the apical foramen (Figs. 7 and 8).

Smaller vessels running between the root canal and the periodontal membrane are relatively common, and almost always these connections consist of a pair of vessels, one large and one small (Figs. 9 and 10).

In multirooted teeth having dentine and cementum "webs" joining adjacent roots (for example, upper molars having joined palatal and distobuccal roots) there is much

anastomosis between the vessel systems of the main root canals. Examples have also been found in which relatively large vessels penetrate the "web" and run to both canals (Fig. 11).

Arteriovenous connections

In many tissues the arterial and venous blood flows are joined, not only by the capillary plexus, but also by direct arteriovenous connections. It is believed that these direct connections play an important part in the local regulation of blood flow.

Because it is almost completely encased in the rigid, unyielding dentine, no significant expansion of the pulp can occur, and it is thought that in acute inflammatory conditions of the pulp mechanisms that would produce swelling in other tissues may result in strangulation of the apical vessels and consequent pulpal necrosis. Arteriovenous shunts might have an important influence upon the outcome of episodes tending to disturb the blood-flow in the pulp, but so far as is known, the demonstration of these structures in the pulp has not previously been reported.

When dealing with larger tissue masses or organs, arteries and veins can be cannulated separately and distinctively coloured injection masses introduced; arteriovenous shunts are well displayed and easily recognized because of the mingling of the differently coloured injection masses. Unfortunately, this method can be not used on extracted teeth and arteriovenous shunts can be identified only by the more laborious process of following in detail the courses of easily identified arteries and veins to see whether connections between them, other than by way of the capillary plexus, can be found. Figs. 12 and 13 show a preparation in which such connections could be demonstrated. They are more readily seen in the large multiple-field photomicrograph; as this can be reproduced here only at a lower magnification the photomicrograph is accompanied by a tracing of the vessel system. The direct connections between arterial and venous systems, short circuiting the capillary plexus, are indicated by dotted lines.

It has not yet been possible to identify these linking vessels in histological sections, and therefore it is not known whether their walls have the specialized structure associated with arteriovenous shunts in other tissues.

The peripheral plexus

The peripheral capillary plexus shows features both of distribution and of pattern that do not appear to have been described previously. Generally, the plexus is present over the whole periphery of the coronal part of the pulp, and extends for a variable distance into the root canals. Within the root, it was often found that the peripheral plexus was present only on one aspect of the canal, whilst on the other aspect there were only the larger vessels running to and from the coronal part of the pulp (Fig. 9).

The peripheral plexus is mainly sub-odontoblastic, but a small number of capillary loops penetrate the odontoblast layer and lie amongst the cells close to the inner aspect of the predentine (Figs. 14 and 15). Many of the peripheral loops have projecting from their outer aspects, short thornlike irregularities (Figs. 16 and 17). At first it was thought that this appearance resulted either from incomplete filling of branches with

the injection mass, or as a result of viewing small loops from the side, so that the two arms of the loop were superimposed. However, careful examination showed that the projections were not attributable to either of these causes. It seems possible that they represent the origins and terminations of loops that formerly were present, and became occluded and lost during the reduction in pulp volume with progressive dentine formation.

Very occasionally, long capillary loops were found projecting outwards from the peripheral plexus and extending far into slender canals in the dentine. These long vascular projections were found mainly directed towards the root bifurcation (Figs. 18 and 19), and may represent part of the rich vascular network between the root vessel systems that had become trapped by physiological secondary dentine formation on the floor of the pulp chamber.

As pointed out by SAUNDERS (1957a) the peripheral plexus consists not only of isolated capillary loops, but also of a rich anastomotic network. This could be clearly demonstrated by photomicrography using a wide lens aperture, so that the depth of field in focus was small (Fig. 5).

DISCUSSION

The methods used in this study permit the direct microscopic examination of Indian ink-filled vessels in cleared specimens. SAUNDERS and others, using a modification of the original suction-injection method (KRAMER, 1951) have preferred a radio-opaque injection mass visualized by microradiography. Both methods have their advantages and disadvantages. Using the radiographic technique the vessels of a considerable thickness of tissue can be rendered sharply in focus, and there is no necessity for clearing the tissue, a process that is sometimes troublesome. However, the method has the disadvantage that special apparatus is necessary, and subsequently considerable magnification of the radiographic image may be called for (although this difficulty may be overcome by the use of X-ray projection microscopy, as described by SAUNDERS, 1957b). Furthermore, if the tissue is not cleared it is not possible to study the vessels directly under the stereoscopic microscope, a procedure which may be helpful in the interpretation of the photographic or radiographic image.

The Indian ink-clearing technique would not be suitable for tissues that are difficult to clear, but no such difficulty is encountered with the tooth. The main disadvantage of the method is the difficulty of obtaining a sharp photographic image of the vessels throughout a thick tissue mass. The dental pulp is rarely so thick as to be troublesome in this respect, and it is felt that, for studies on the pulp, the Indian ink-clearing technique is usually to be preferred. Furthermore, the method has the advantage that although the tissues are unstained, the odontoblast layer and inner margin of the predentine can be seen. Thus, the relationship of the peripheral capillary loops to the adjacent structures can be determined.

The studies reported here confirm and amplify those reported previously (KRAMER, 1951; RUSSELL and KRAMER, 1956; SAUNDERS, 1957a,b). Of particular interest is the demonstration of arteriovenous connections, the complexity of the vessel system in multi-rooted teeth, and the tendency for the capillary plexus to be present on only one

aspect of the root canal. This distribution of the capillary plexus appears to be related to the distribution of odontoblasts, for in adult teeth it is often noticeable that the odontoblast layer also is present on only one side of the canal, usually the outer side, and the absence of odontoblasts and the absence of the peripheral capillary plexus coincide. Comparing the results of the present study with recent studies on the distribution of the vessels in the pulps of rodent incisors (ADAMS, 1959) it is clear that there are substantial differences. In particular, the peripheral plexus of the pulp in man is quite unlike the peripheral plexus in the rodent incisor; this difference may be of importance when animal experiments are undertaken to determine the pulp response to operative procedures and filling materials.

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FIG. 3. Composite photomicrograph of the injected pulpal vessels in a normal upper second premolar from a female aged 26 years. Serial photomicrographs of overlapping fields have been taken at high magnification, and from these the composite picture has been assembled (a) shows one side of the pulp chamber and the coronal part of the root canal. (b) shows the rest of the root canal of the same tooth. ×26.

3 a   b
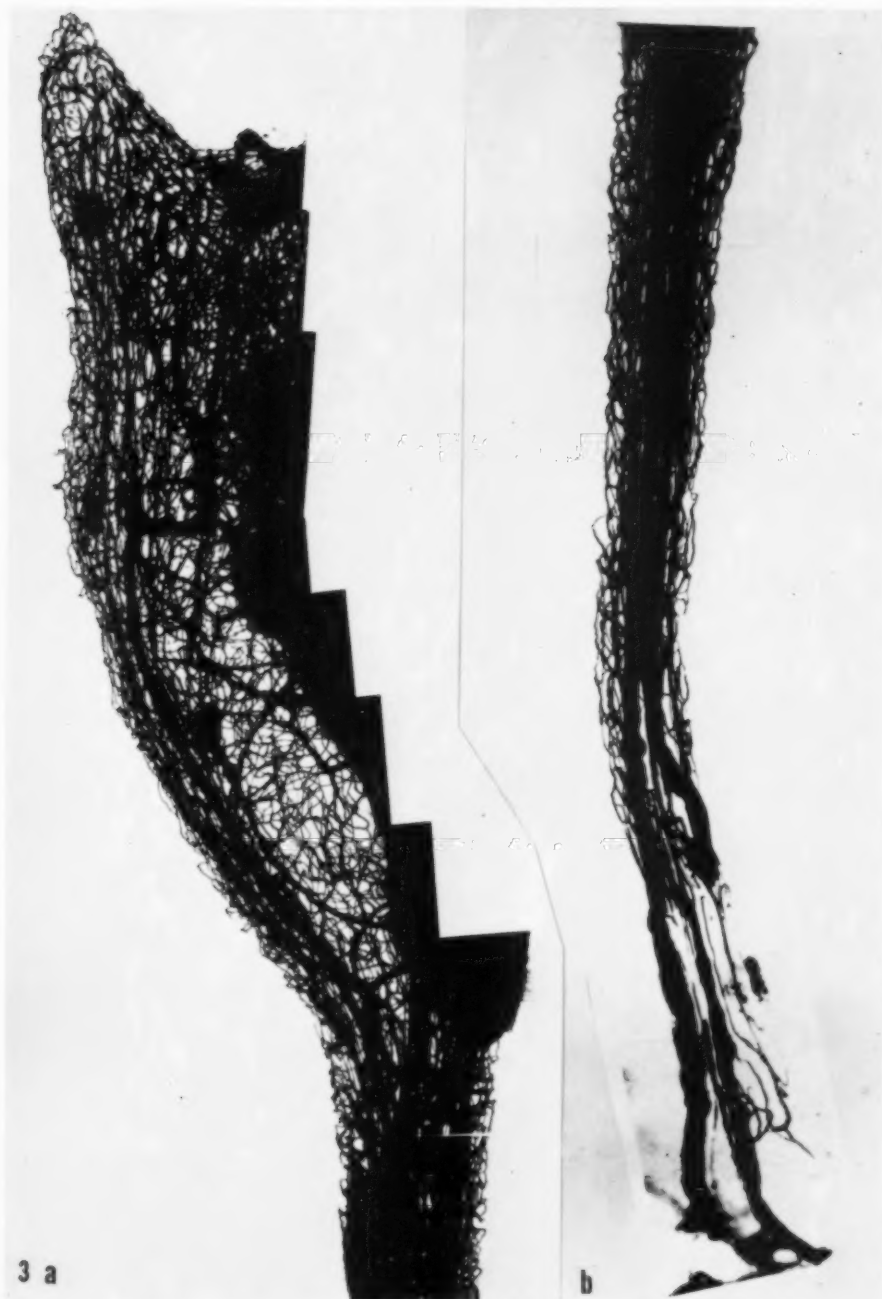
PLATE 1

PLATE 2
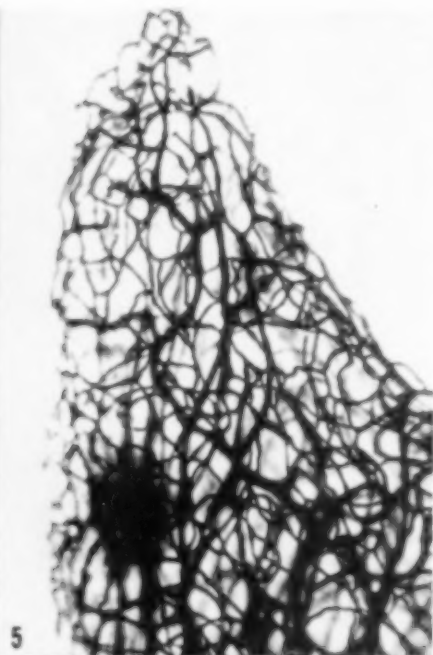
FIG. 4. Single photomicrograph showing the bloodvessels in part of the pulp chamber and root canals of a lower first permanent molar from a male aged 21 years. ×14·5.

FIG. 5. Pulp cornu, same premolar as shown in Fig. 3. By using a wide lens aperture the vessels beneath the superficial plexus have been thrown out of focus, thus facilitating the study of the superficial network. ×57.

FIG. 6. Complex vessel pattern in the roots of a lower second deciduous molar from a girl aged 5 years. ×16.

FIG. 7. Part of the root of an upper second deciduous molar. A large vessel passes into the root canal from the bifurcation region, and the venous drainage of this root canal is mainly towards the bifurcation area. ×20.

FIG. 8. Lower first premolar tooth. The buccal and lingual sides of this tooth were trimmed on a microtome after the pulpal vessels were injected. In (a) the trimmed tooth is seen on edge, with the apex below. A large vessel is seen passing through the hard tissues about halfway up the root. In (b) the same specimen has been rotated through 90°, and it can be seen that the main venous drainage of the pulp is via the vessel perforating the wall of the root canal. ×12.

FIG. 9. Buccal root of an upper first permanent molar tooth. Six groups of vessels connect the root canal with the periodontal membrane. Note also, in the coronal half of the root canal, the peripheral capillary plexus present only on the outer aspect. ×14·5.

FIG. 10. Higher magnification of part of the specimen shown in Fig. 9. In each case, the channels connecting the root canal (left) with the periodontal membrane (right) contain a pair of vessels, one much larger than the other. The smaller vessels resemble arteries, and the larger vessels resemble veins. ×83.

FIG. 11. Lower second deciduous molar. A large vessel enters the "web" between the main canals in one root, and joins the vascular systems of both canals. ×13·5.

PLATE 3

PLATE 4

13
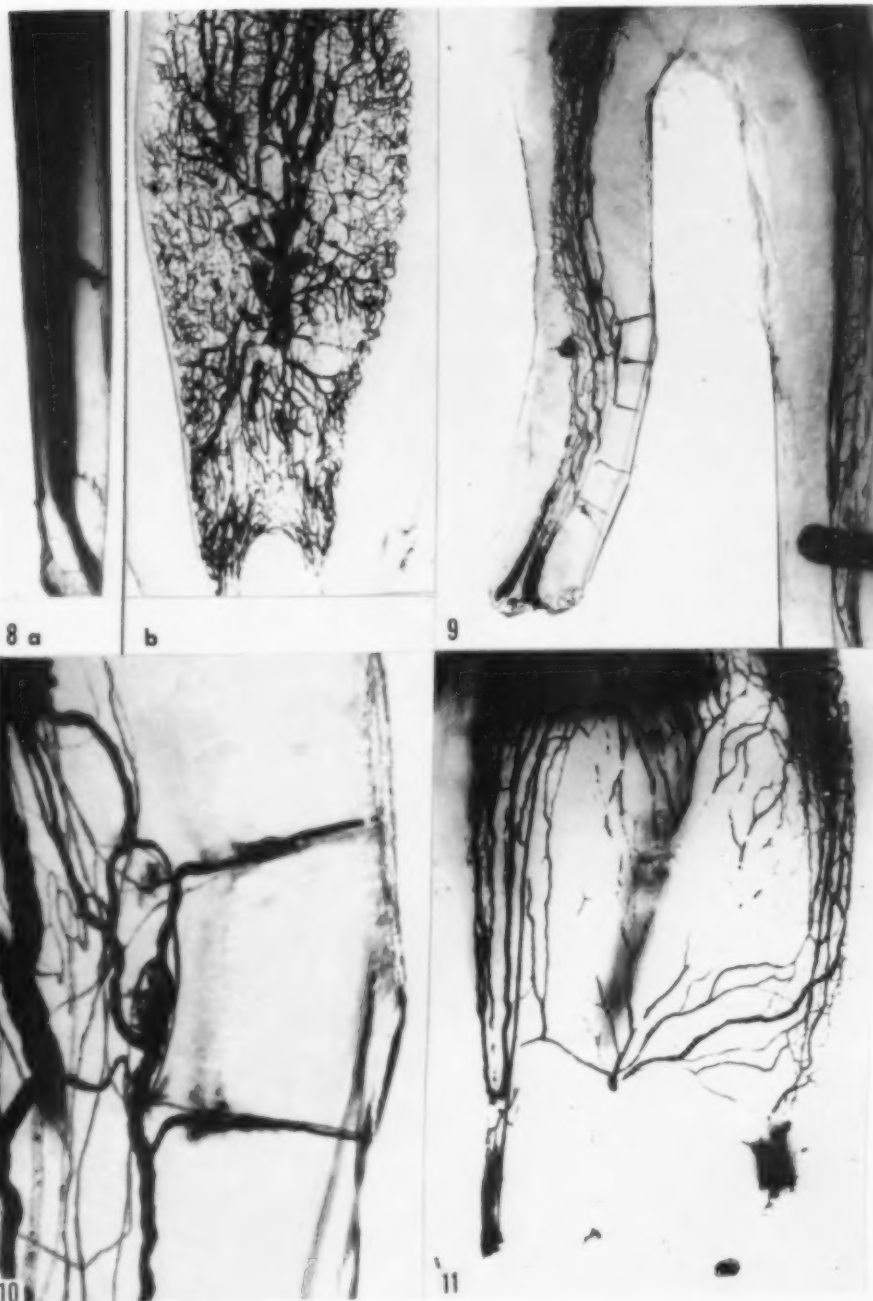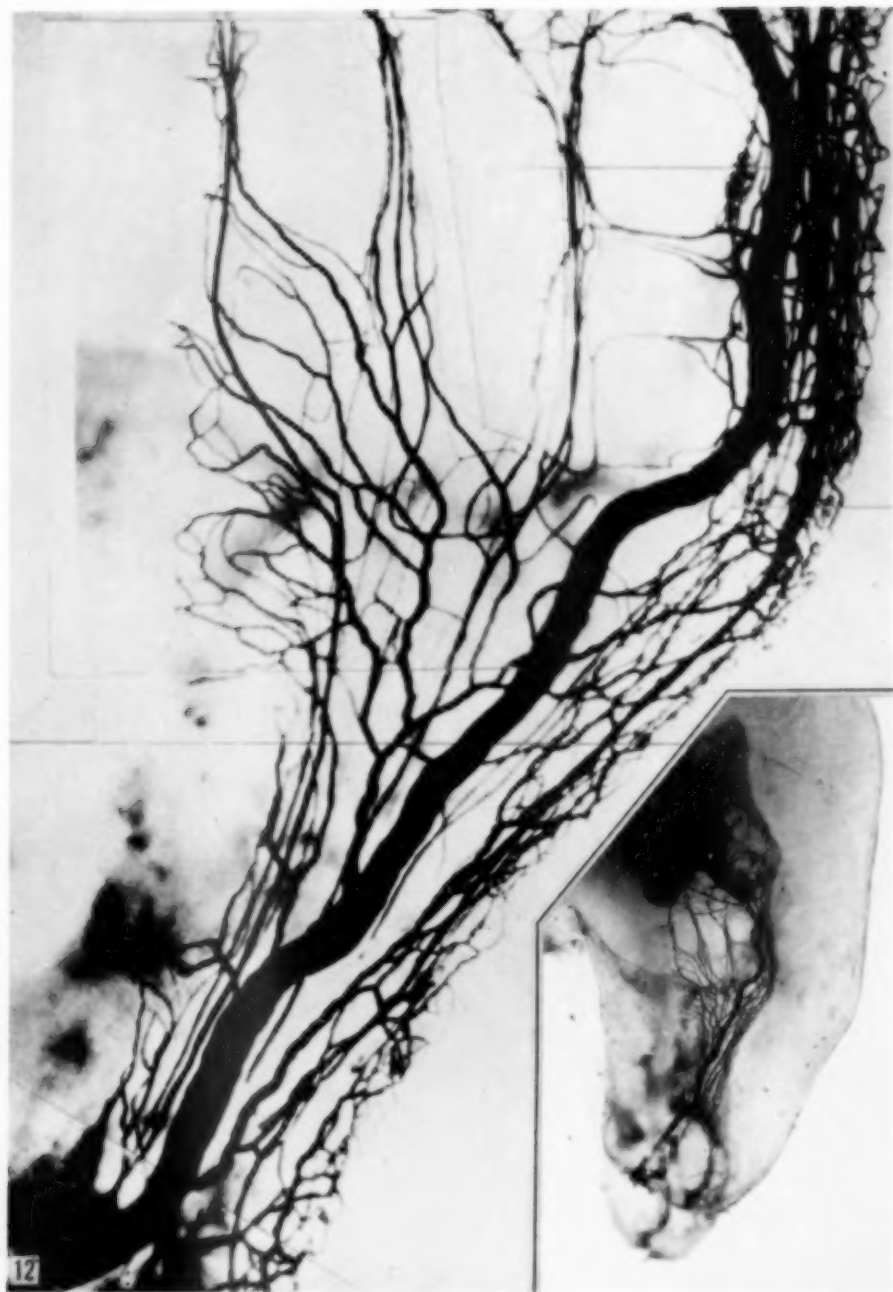
FIG. 12. Composite photomicrograph showing part of the vessel system in a buccal root of an upper first permanent molar tooth. ×40. The inset shows the same specimen at a lower magnification. ×6.

FIG. 13. Tracing of the vessels shown in Fig. 12. Working from the composite photomicrograph, and re-examining the original specimen at the same time, the connections of most of the vessels have been traced. The arterial system is shown in red and the venous system in black. It can be seen that there are connections between the arterial and venous systems before the peripheral capillary plexus. These arterio-venous connections are shown by dotted lines in the tracing.

FIG. 14. A typical part of the peripheral capillary plexus. Part of the dentine above and below the field of view was removed, so that the relationship of the vessels to the pulpodentinal junction could be seen more clearly. Whilst the plexus is mainly sub-odontoblastic, a few loops project into the odontoblast layer. ×88.

FIG. 15. Composite photomicrograph showing another part of the peripheral plexus. ×58.

PLATE 5

PLATE 6
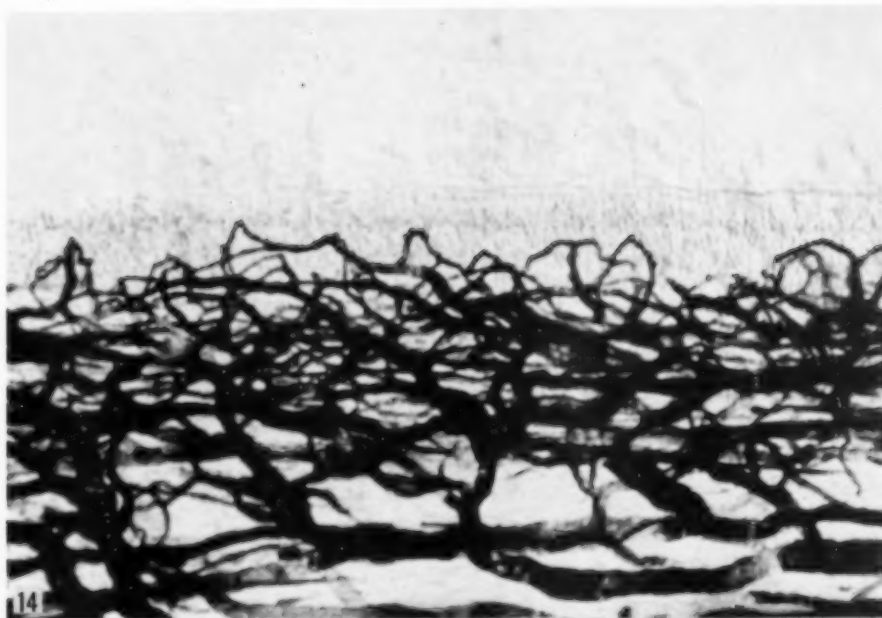
Fig. 16. Loops of the peripheral capillary plexus, in a lower canine tooth from a female aged 60 years, showing the thorn-like projections.   ×150.

Fig. 17. Vessels of a root canal, upper third molar from a female aged 28 years. On the right there are thorn-like projections and acute-angled loops that may result from progressive attenuation and loss of the broader loops.   ×75.

Fig. 18. Roots of an upper first deciduous molar tooth. A long, slender vessel loop runs inwards towards the bifurcation area. Two other, smaller, loops are also seen.   ×20.

Fig. 19. Higher magnification of the loop shown in Fig. 18.   ×60.

RELATION OF THE FLUORIDE CONTENT OF HUMAN BONE TO ITS CHEMICAL COMPOSITION

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Abstract—A comprehensive study is presented on the composition of human bone over a wide range of skeletal fluoride concentrations. Thus, sixty-nine samples of human bone, comprising iliac crest, rib and vertebra obtained from twenty-three individuals 26–90 years of age who had consumed drinking water containing up to 4·0 p.p.m. fluoride for 10–87 yr prior to demise, were analysed for calcium, phosphorus, magnesium, sodium, potassium, carbon dioxide and citrate. The percentage of calcium and phosphorus in the dry, fat-free bones was normal although they contained as much as 0·4% fluoride.

No relation was apparent between the fluoride present in bone ash over a ten-fold range in concentration and its calcium or phosphorus content. With increased levels of fluoride in the ash (0·08–0·8%) there was a slight increase in magnesium and a decrease in carbon dioxide. The citrate content decreased markedly with increased fluoride. A slight decrease in sodium and little or no change in potassium were noted. The data support the hypothesis that fluoride is deposited in mature bone largely at the expense of surface limited ions.

IN previous publications from this laboratory (ZIPKIN, McCLURE, LEONE and LEE, 1958; McCLURE, McCANN and LEONE, 1958) data were presented demonstrating significant increases in the fluoride content of human bones as a result of a prolonged ingestion of fluoride in drinking water. Although above normal in fluoride content, no related changes in the bone tissues were apparent on histologic examination (GEEVER, LEONE, GEISER and LIEBERMANN, 1958). Previous information on the effect of increased fluoride on the chemical composition of human bones is quite limited. In one case reported by WOLFF and KERR (1938) excessive fluoride ingestion increased bone-ash fluoride up to 1·07% without, however, affecting the calcium, phosphorus or carbon dioxide content of the bones. GLOCK, LOWATER and MURRAY (1941) and CALL and GREENWOOD (1958) also reported no changes in ash, calcium and phosphorus in human bones containing up to 0·69 and 0·15% fluoride in the ash respectively. The former investigators also found no change in magnesium. McCLURE, McCANN and LEONE (1958) recently reported an increase in carbon dioxide, a reduction in magnesium and phosphorus but no change in the calcium content of the ash of a number of bones of one individual containing up to 0·97% fluoride.

Numerous experiments involving a variety of animal species have related the fluoride content of bone to a number of chemical constituents (McCLURE, 1939; McCANN and BULLOCK, 1957; WEIDMANN, WETHERALL and WHITEHEAD, 1959). In these studies, unusually large quantities of fluoride ingested in food and water had produced relatively excessive deposits of bone-fluoride over a short space of time. While these

experiments provide valuable information, the results are not generally applicable to conditions surrounding a prolonged human exposure to low levels of water-borne fluoride.

Continuing our studies on the effects of water-borne fluoride, the current report is concerned with the ash, calcium, phosphorus, sodium, potassium, magnesium, carbon dioxide, and citrate of human skeletal tissue in relation to its fluoride content. Analyses were made of sixty-nine bone specimens from twenty-three individuals, selected from the previous studies (ZIPKIN *et al.*, 1958; GEEVER *et al.*, 1958) who were from 26 to 90 years of age and had consumed drinking water containing up to 4·0 p.p.m. fluoride for 10–87 yr prior to death.

METHODS

Dry, fat-free bone samples were prepared as previously described (ZIPKIN *et al.*, 1958) for the determination of carbon dioxide (DEAKINS and BURT, 1944) and citrate (ZIPKIN and McCLURE, 1949; ZIPKIN and PIEZ, 1950). The bone ash was analysed for calcium after double precipitation as the oxalate (KOLTHOFF and SANDELL, 1952) to ensure good separation of magnesium. The precipitate was fired at 1000°C for one-half hour in a muffle, treated with hydrogen fluoride and evaporated to dryness on a steam bath, ignited to dull red heat over a bunsen flame and weighed as calcium fluoride. Phosphorus was determined on the ash (GEE and DIETZ, 1953) and magnesium was determined on the filtrate from the calcium analysis by double precipitation as magnesium ammonium phosphate (KOLTHOFF and SANDELL, 1952). Sodium and potassium were determined by internal standard flame photometry and interferences of calcium and phosphorus in the samples were overcome by adding similar amounts to the standards.

RESULTS

The quantity of fluoride in the bone ash varied ten-fold, i.e. from 0·07 to 0·80%. A straight line function best describes the relation of the fluoride in the drinking water to that in the bone ash (ZIPKIN *et al.*, 1958).

The bone analyses are presented on an ash basis in Table 1 where the results are grouped according to the amount of fluoride in the individuals' drinking water. The major bone constituents, calcium and phosphorus, in the bone ash and the calcium : phosphorus ratio remained unaffected by fluoride. A comparison of individual bones indicates that some slight increase in their ash content may be ascribed to fluoride. As the fluoride content of the drinking water increased from <1·0 up to 4·0 p.p.m., the ash of the iliac crest increased from 53·51 to 58·62%, of the rib from 56·04 to 57·38% and of the vertebra from 49·82 to 52·33%.

As the fluoride in the bones increased, magnesium increased in the ash of all bones from 0·50% to a maximum of 0·57, 0·60 and 0·61% for the iliac crest, rib and vertebra respectively. A slight but consistent decrease in sodium occurred in bones, i.e. in the iliac crest from 0·73 to 0·70%, in the rib from 0·79 to 0·63% and in the vertebra from 0·73 to 0·68%. In general the potassium concentration of the bone ash decreased slightly with increasing concentrations of fluoride.

TABLE 1. ASH (DRY, FAT-FREE BASIS), FLUORIDE, CALCIUM, PHOSPHORUS, MAGNESIUM, SODIUM, POTASSIUM, CITRATE AND CARBONATE CONTENT (ASH BASIS) OF SELECTED HUMAN BONES

Bone sample	No.	F (%)	Ash (%)	Ca (%)	P (%)	Ca:P*	Mg (%)	Na (%)	K (%)	Na:K*	CO$_2$ (%)	Cit (%)
					<1·0 p.p.m. F in drinking water							
Iliac crest	6	0·08±0·01	53·51±1·44	38·80±0·11	17·45±0·05	2·22±0·01	0·50±0·02	0·73±0·02	0·09±0·01	8·58±0·94	5·83±0·21	2·23±0·13
Rib	5	0·08±0·01	56·04±1·16	38·82±0·15	17·45±0·08	2·22±0·01	0·50±0·01	0·79±0·02	0·09±0·01	9·12±0·98	5·41±0·23	1·92±0·16
Vertebra	6	0·10±0·01	49·82±1·29	37·82±0·23	17·52±0·10	2·16±0·02	0·50±0·01	0·73±0·02	0·18±0·03	4·30±0·52	5·31±0·15	1·95±0·09
Sternum	5	0·13±0·02	48·59±1·95	38·39±0·21	18·05±0·22	2·14±0·02	0·46±0·01	0·67±0·03	0·06±0·01	13·77±2·62	4·67±0·10	1·86±0·16
					1·0 p.p.m. F in drinking water							
Iliac crest	4	0·22±0·03	58·18±0·69	37·76±0·63	17·62±0·15	2·15±0·05	0·52±0·02	0·72±0·02	0·06±0·01	13·19±2·75	5·60±0·44	1·95±0·05
Rib	4	0·24±0·04	54·77±0·62	36·67±0·75	17·57±0·07	2·09±0·04	0·50±0·02	0·69±0·02	0·10±0·03	8·18±1·73	5·30±0·11	1·67±0·19
Vertebra	4	0·34±0·06	47·24±1·25	36·15±0·81	17·73±0·12	2·04±0·06	0·55±0·03	0·68±0·03	0·11±0·01	6·29±0·86	4·43±0·46	1·45±0·14
					2·6 p.p.m. F in drinking water							
Iliac crest	7	0·40±0·09	58·92±0·61	38·12±0·33	17·05±0·11	2·24±0·02	0·58±0·02	0·72±0·03	0·08±0·01	9·50±1·38	5·08±0·18	1·75±0·11
Rib	7	0·41±0·09	57·47±1·16	38·08±0·32	17·33±0·11	2·20±0·02	0·57±0·02	0·69±0·02	0·07±0·01	10·06±1·20	5·08±0·24	1·64±0·07
Vertebra	8	0·56±0·11	49·79±0·75	37·24±0·40	17·59±0·23	2·12±0·02	0·63±0·02	0·72±0·03	0·17±0·02	4·44±0·44	4·24±0·23	1·36±0·08
					4·0 p.p.m. F in drinking water							
Iliac crest	4	0·69±0·09	58·62±1·12	38·41±0·38	17·74±0·39	2·17±0·06	0·57±0·02	0·70±0·04	0·08±0·02	9·24±1·76	5·20±0·39	1·56±0·32
Rib	4	0·70±0·09	57·38±2·65	38·36±0·32	17·74±0·29	2·16±0·05	0·60±0·02	0·68±0·03	0·10±0·04	8·73±1·84	5·19±0·33	1·39±0·23
Vertebra	4	0·80±0·13	52·33±3·12	37·75±0·18	17·49±0·24	2·16±0·04	0·61±0·04	0·68±0·03	0·12±0·02	5·94±1·00	4·55±0·44	1·30±0·12

All estimates of error expressed as standard error.   * Based on means of individual ratios.

With increasing fluoride the carbon dioxide decreased in all bones. Thus in the iliac crest the decrease was from 5·83 to 5·20%, in the rib from 5·41 to 5·19% and in the vertebra from 5·31 to 4·55%.
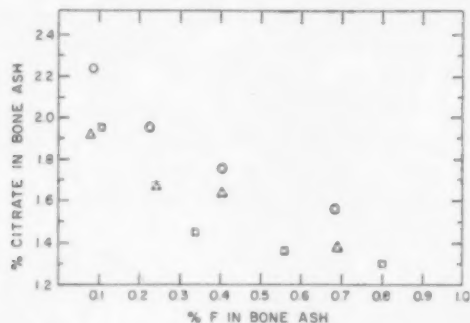
FIG. 1. Relation of fluoride to citrate in bone expressed on an ash basis. ○ Iliac crest; △ Rib; □ Vertebra.

The most striking effect of the increase in bone fluoride was a 30 per cent reduction of citrate in the bones of those individuals using water containing 4·0 p.p.m. fluoride. Thus, the citrate content of the ash of the iliac crest decreased from 2·23 to 1·56%, of the rib from 1·92 to 1·39% and of the vertebra from 1·95 to 1·30%. The data on the citrate content of the bone expressed on an ash basis are shown graphically in Fig. 1.

DISCUSSION

A summary of our data on ash, calcium and phosphorus, along with other similar data for normal as well as highly fluorosed human bones, is presented in Table 2. It is apparent that bones containing over 0·4% fluoride (0·8% in the ash) show no

TABLE 2. COMPARISON OF COMPOSITION OF HUMAN SKELETAL TISSUES EXPOSED
TO NORMAL (<1·0 p.p.m.) AND FLUORIDE WATERS

Water-borne fluoride exposure	Dry, fat-free bone					Bone ash	
	Fluoride (%)	Ash (%)	Calcium (%)	Phosphorus (%)	Ca : P	Calcium (%)	Phosphorus (%)
normal*	0·045	58·20	21·70	9·95	2·18	37·29	17·09
normal†	0·058	57·21	22·04	9·89	2·23	38·53	17·28
<1·0 p.p.m.	0·049	52·82	20·36	9·23	2·20	38·48	17·47
1·0 p.p.m.	0·147	53·40	19·72	9·42	2·09	36·86	17·64
2·6 p.p.m.	0·247	55·40	20·97	9·62	2·19	37·81	17·32
4·0 p.p.m.	0·409	56·11	21·44	9·91	2·16	38·17	17·66
8·0 p.p.m. †	0·556	64·91	24·16	10·55	2·27	37·22	16·25

* CALL and GREENWOOD (1958).
† MCCLURE, MCCANN and LEONE (1958).

change in calcium and phosphate content. The increase in calcium and phosphorus in the dry, fat-free bones containing 0·6% fluoride is a reflection of the increased ash content which had a normal calcium but a slightly lower phosphorus content.

The present *in vivo* data confirm other *in vitro* findings that fluoride replaces carbon dioxide (Neuman *et al.*, 1950), inhibits citrate uptake (Armstrong and Singer, 1956), and has no influence on calcium or phosphorus exchange (Neuman *et al.*, 1950). An explanation for this result may reside in the fact that sodium, potassium, magnesium, carbon dioxide and citrate are presumed to be confined to the surfaces of the apatite bone crystals (Neuman and Neuman, 1953, 1958; Hendricks, 1952), although in these regards there may be some uncertainty concerning the position of magnesium (Klement, 1936) and carbon dioxide (McConnell, 1952; Buchanan and Nakao, 1958) in the crystal.

As regards magnesium, the apparent increase which accompanies fluoride deposition may be explained as due to its affinity for fluoride (McCann and Bullock, 1957). Thus the increase in magnesium would be secondary to fluoride deposition.

The observed reduction in citrate in these bones, concomitant with the increase in bone fluoride, suggests that citrate which is assigned to positions on the crystal surface, may be replaced by fluoride through ion-exchange processes.

Similarly it may be noted that due to cessation of bone growth, exchange reactions of bone surfaces would predominate in mature and aged bone. Advanced age at the time of initial exposure to fluoride thus favours the deposition of most of the fluoride at the expense of surface limited ions.

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THE EFFECTS OF STRESS ON THE SUBMAXILLARY GLANDS OF YOUNG ADULT MALE RATS

SELECTED HISTOCHEMICAL OBSERVATIONS

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Abstract—Twenty-seven young adult male rats were injected intra-muscularly with 0·5 ml turpentine per day and sacrificed immediately after injection, and 1, 3, 6, 24, 48, 72, 96 and 120 hr after the start of the injections. The adrenal glands were examined histologically for evidence of stress. The submaxillary glands were stained with haematoxylin and eosin, the periodic acid–Schiff technique with 2-hour and 18-hour malt diastase digestion, the methyl green–pyronin method with ribonuclease digestion and the calcium–cobalt technique for alkaline phosphatase.

During the alarm reaction the submaxillary glands showed a decrease in RNA, a decrease in the granularity of the acini, a decrease in interacinar connective tissue and an increase in granularity of the duct cells. The ducts stained more PAS-positive than the acini. A highly insoluble, heat-resistant, diastase-fast, PAS-positive material was present in the duct cells. During the stage of resistance, the duct cells showed a decrease in granules with little acinar change. No observable difference in alkaline phosphatase activity was noted.

In the past two decades, much attention has been focused on the effects of the general adaptation syndrome on various organs and biologic systems.

Among the organs studied, salivary glands have been shown to react in the first phase of the general adaptation syndrome; the alarm reaction (Ehrich and Seifter, 1948; Shklar and Glickman, 1953). These investigations were limited to histologic observation. The present study was undertaken in order to investigate the response of the submaxillary glands to stress by means of selected histochemical techniques and to correlate these observations with the previously reported morphologic alterations.

Grossly observed changes in stomach mucosa and histologic aberrations of the adrenal cortex were used as an index of the response of the animals to stress.

MATERIALS AND METHODS

Twenty-seven Sprague Dawley male albino rats weighing an average of 320 g at the start of the experiment were kept, two animals per cage, under identical conditions for at least 1 month prior to the initiation of the stressor. All animals were given food and water *ad libitum*. Upon initiation of the stress stimulus, the animals were divided at random into two groups: a control group of nine animals and an experimental group of eighteen animals. 0·5 ml of commercial turpentine, one of the chemical inducers of non-specific stress (Selye, 1950) was injected daily intramuscularly into

the hind limb of the experimental group. Except for injection, the control group received as much handling as the experimental group.

Animals were sacrificed with ether in groups of two experimental and one control in accordance with the following time schedule: immediately after injection, and 1, 3, 6, 24, 48, 72, 96 and 120 hr after the start of the injections. The submaxillary glands were removed immediately, one portion was fixed in cold acetone and the remainder in 10% formalin. The adrenals were then removed and placed in 10% formalin. The stomach was dissected out for gross observation.

After adequate fixation, the submaxillary glands and adrenals were removed from the 10% formalin, dehydrated and embedded in paraffin wax. All tissues were sectioned at 5–7 μ and stained with haematoxylin and eosin. Alternate sections of the submaxillary glands were stained with the periodic acid–Schiff technique with malt diastase digestion for 2 hr and 18 hr with a buffer control, and the methyl green–pyronin method with ribonuclease digestion with a buffer control. Those portions of the submaxillary glands fixed in cold acetone were double embedded according to the method of GOMORI (1952) and stained with haematoxylin and eosin and the calcium–cobalt method for alkaline phosphatase. The preceding histochemical techniques follow those outlined by PEARSE (1953). Control and experimental sections were stained simultaneously in all cases.

EXPERIMENTAL RESULTS

Gross observation

Redness of the gastric mucosa was noted in animals after 48 and 72 hr of stress duration. Areas of redness and ulceration were seen in the gastric mucosa of animals subjected to 96 and 120 hr of stress.

Histologic observations

Adrenal glands. The adrenal glands of the control animals (Fig. 1) appeared histologically similar to the adrenal glands of normal rats (PAULY, 1957). Until 6 hr after stress initiation no significant changes in adrenal morphology were observed. However, in those animals sacrificed after 6 hr of stress, a notable decrease in the lipid content of the fasciculata cells was observed. A peak response was obtained in animals subjected to 24 and 48 hr of stress. The glomerulosa appeared normal, but within the fasciculata large areas of necrosis could be found, usually located in the outermost portion of the zone. Throughout the width of the discernible fasciculata large areas were seen in which the cells showed marked lipid loss (Fig. 2) and lipid reaccumulation. The zone delineation with respect to the fasciculata–reticularis border was indistinct. Hypertrophy and hyperplasia of the fasciculata cells were noted, the former appearing mostly in the outer fasciculata and the latter in the innermost layer of this zone. At the medulla–cortex boundary, extreme hyperaemia was observed.

Submaxillary glands. During the entire experimental period, the submaxillary glands of the control animals (Fig. 3) appeared similar to the accepted histologic description of rat submaxillary glands (STORMONT, 1932).

Until 24 hr after stress initiation no significant changes were noted. At this time, however, a decrease in basophilia in the acinar cells accompanied by a decrease in granules was noted. Many vacuolated cells were seen in both ducts and acini. The secretory ducts showed great variation in the size of the granules. There also appeared to be a decrease in connective tissue elements surrounding the acini. 48 hr and 72 hr after stress initiation a progressive decrease in basophilia in the acinar cells and a continued decrease in granular content were noted. The decrease in connective tissue elements remained constant. The greatest changes occurred in the duct cells. The luminal borders of the cells of the secretory ducts appeared to be composed of a markedly eosinophilic, homogeneous accumulation which contained varying numbers of highly refractive granules which varied further with respect to size. In some speci-mens, granular detail was not resolvable (Fig. 4). After 96 hr and 120 hr of stress, the acinar changes were slight. Aside from an increase in vacuolization there was little notable change. The secretory duct cells appeared vacuolated at the luminal borders and almost devoid of granules.

Histochemical observations

Alkaline phosphatase. The method employed did not show any difference in the localization of alkaline phosphatase activity. In both control and stressed animals, the areas around the acini and the smaller ducts were intensely positive. No attempt at quantitative measurement was made.

Periodic acid–Schiff technique with diastase digestion. In all control animals the results were the same. The acini stained positively, while both large and small ducts stained for the most part negatively, exhibiting no colour or slight colour at the luminal borders of the cells (Fig. 5). After 2 hr of diastase digestion, the intensity of the PAS reaction was decreased equally throughout the section. Eighteen hour diastase digestion resulted in an overall decrease until practically no PAS-positive material remained in the section. (Figs. 7 and 9).

In those animals sacrificed 24 and 48 hr after stress initiation, the ducts appeared to stain more PAS-positively than did the acini. A gradual increase in their staining intensity was noted with time. Thus, in the 24-hour stress group, the ducts stained less intensely, while in the 48-hour stress group, almost all the ducts exhibited marked fuchsinophilia (Fig. 6). Digestion with malt diastase for 2 hr caused an overall de-crease in the staining intensity, which appeared greater in the acini than in the duct cells. However, 18-hour digestion revealed intensely positive duct cells only (Figs. 8 and 10). In those animals sacrificed more than 72 hr after stress initiation, the stain-ing response described previously was decreased. In the 5-day (120-hour) stress group, the ducts again appeared to stain less positively than the acini and in some cases were negative.

Ribonucleic acid. In the control animals, the RNA appeared as a darkly staining substance surrounding the granules in the acini. After ribonuclease digestion, the granules stood out distinctly in a relatively clear cytoplasm. In those animals sub-jected to stress for 24–48 hr, the granules appeared almost as distinct as in those sections of the control glands which had been subjected to ribonuclease digestion.

DISCUSSION

Extent of stress

All of the stressed animals in our experimental group exhibited adrenal–cortical changes similar to those described by BAKER (1954), GREEP and DEANE (1949) and SELYE and STONE (1950) as indicative of cortical response to general systemic stress. Using these descriptions as a guide, it may be stated that the alarm reaction began 6 hr after stress initiation and lasted 24–48 hr. The second phase or stage of resistance began about 48 hr after induction of stress and lasted for the duration of the experiment.

These observations agree basically with those outlined by SELYE (1950). However, in our animals, the alarm reaction manifested itself somewhat more slowly. This may be due either to slow absorption of the turpentine or may indicate that turpentine is not as severe a stress inductor as those used by other investigators (SELYE, 1950).

Effects of stress on the submaxillary glands

Morphological effects. The response of the submaxillary glands of the rat during the alarm reaction have been reported previously (EHRICH and SEIFTER, 1948; SHKLAR and GLICKMAN, 1953). The decrease in basophilia in the acinar cells, the decrease in granules and the decrease in connective tissue elements that these investigators noted were also seen in our animals. However, by increasing the duration of the stress stimulus, we have noted that not only the acini but also the secretory ducts appear to be affected, the latter structures showing very marked changes. Morphological alteration in the granular content at the luminal borders of these cells in the alarm reaction was followed in the stage of resistance either by an emptying of these cells, a discharge of the granules or vacuolization of the luminal borders. Similar alteration of both acini and duct cells has been reported previously but not in connexion with the general adaptation syndrome. It has been shown by BIXLER, MUHLER, WEBSTER and SHAFER (1957), BIXLER, MUHLER and SHAFER (1957), BIXLER, WEBSTER and MUHLER (1957) and SREEBNY (1954) that hypophysectomy results in a decrease in granules in both the acini and the cells of the secretory ducts. SHKLAR, GLICKMAN and TURESKY (1958) found that cortisone injections resulted in a diminution of the interacinar tissue. It is of interest to note that the majority of studies dealing with the activity of the secretory duct granules are concerned with the relationship between these granules and the proteolytic activity of the gland. BIXLER, MUHLER and SHAFER (1957), SREEBNY, MEYER and BACHEM (1953) and SREEBNY *et al.* (1956) seem to be in accord with the hypothesis that these granules are not only related to the proteolytic activity of the salivary glands but are also responsible for it. If this be the case, our data suggest that an increase in proteolytic activity occurs during the alarm reaction followed by a sharp decrease during the stage of resistance.

Histochemical effects. Any alteration in histologic structure of an organ must be reflected in an aberration from normal function of that organ. The histochemical techniques employed in this investigation were used in order to uncover any alteration in the normal metabolic activity of the submaxillary gland.

As stated previously, no apparent difference in the localization of alkaline phosphatase activity was noted in any of the animals studied. The technique employed, while specific for phosphatase, has as a drawback a high rate of diffusion of end product to other sites within the cell (BARTER, 1954; JOHANSEN and LINDSTROM-LANG, 1953). By allowing only short incubation (1 hr) in the substrate, it was possible to minimize diffusion artifacts (EMMEL, 1946). GOMORI (1950) stated that it was possible to quantitate results achieved in this manner, but since differences, if any, were minimal, this was not attempted.

The results obtained using the periodic acid–Schiff method on submaxillary gland sections of control animals agree with those of HILL and BOURNE (1954) and SHKLAR, GLICKMAN and TURESKY (1958) in that the acini stain positively and the ducts for the most part negatively. Malt diastase digestion has been shown by SCOTT and CLAYTON (1950) to cause an overall decrease in the stain intensity and complete removal of glycogen when the section is incubated for 1 hr in a 1% solution at 37° C. Two-hour digestion in this solution revealed little change, indicating that the positive reaction was not primarily due to the presence of glycogen. Drastic digestion with malt diastase for 18 hr revealed almost complete removal of all positive material with the exception of a slight residue in some of the smaller ducts. In our experimental animals, at the height of the alarm reaction, the ducts appeared to stain more intensely than the acini. Two-hour digestion did not appreciably decrease the ductal reaction, and after 18 hr of digestion only the ducts stained positively.

From the foregoing, it appears that during the alarm reaction a rapid accumulation of diastase-fast, PAS-positive material occurs in the rat submaxillary gland. SHKLAR, GLICKMAN and TURESKY (1958), using cortisone injections in young male and young female mice, noted an unaltered duct response coupled with an increase in the staining intensity of the serous cells, due to the presence of diastase-fast, PAS-positive material. Our results, using a chemical stressor in young adult male rats revealed the presence of a highly insoluble, heat resistant, diastase-fast, PAS-positive material in the intralobular duct system only. This may represent a mucopolysaccharide or more probably a mucoprotein. Drastic treatment of fixed tissue such as was employed in our experiment, as evidenced by our results, probably results in more than the mere removal of glycogen.

Since no biochemical determination was employed, we cannot speculate on the nature of this material other than to say that it contains the 1-2 glycol group necessary for it to react with the PAS reagent. From the evidence offered by the control animals we may further state that this substance is present in a very small percentage of the normal submaxillary gland tissue, but appeared greatly increased in our experimental animals in association with the induced state of stress. This is consistent with the observation by BABKIN (1950) that the salivary gland may not react as a whole, but that various portions may react independently. The disappearance of this fuchsinophilic material from the duct system during the stage of resistance suggests that it may represent a specific response of the rat submaxillary gland duct cells to the onset of stress.

EHRICH and SEIFTER (1948) and SHKLAR and GLICKMAN (1953) assumed that the decrease in basophilia in the acinar cells during the alarm reaction was due to a decrease

in ribonucleic acid in those cells. The results obtained with this method tend to support these findings in that at the time of decreased basophilia (alarm reaction) less RNA was present in the basal cytoplasm of the acinar cells of the submaxillary gland.

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FIG. 1. Part of the fasciculata of the adrenal gland of a control animal. Note the regular cord-like arrangement and the vacuolated appearance of the cells. Haematoxylin and eosin. ×100.

FIG. 2. Part of the fasciculata of the adrenal gland of an animal sacrificed 24 hr after stress initiation. Note the irregular arrangement of the cells and the appearance of the cytoplasm as compared to Fig. 1. Haematoxylin and eosin. ×100.

FIG. 3. Submaxillary gland of a control animal. Note the granularity of the ducts. Haematoxylin and eosin. ×100.

FIG. 4. Submaxillary gland of an animal sacrificed 96 hr after stress initiation. Note the decreased basophilia and decreased granularity of acini and ducts. Haematoxylin and eosin. ×100.

FIG. 5. Submaxillary gland of a control animal showing the localization of PAS-positive material. Black arrows indicate PAS-negative ducts and the white arrow a PAS-positive duct. Periodic acid–Schiff. ×100.

FIG. 6. Submaxillary gland of an experimental animal sacrificed 48 hr after stress initiation. The black arrow indicates a negative duct and the white arrows positive ducts which stain more intensely than the surrounding acini. Periodic acid–Schiff. ×100.
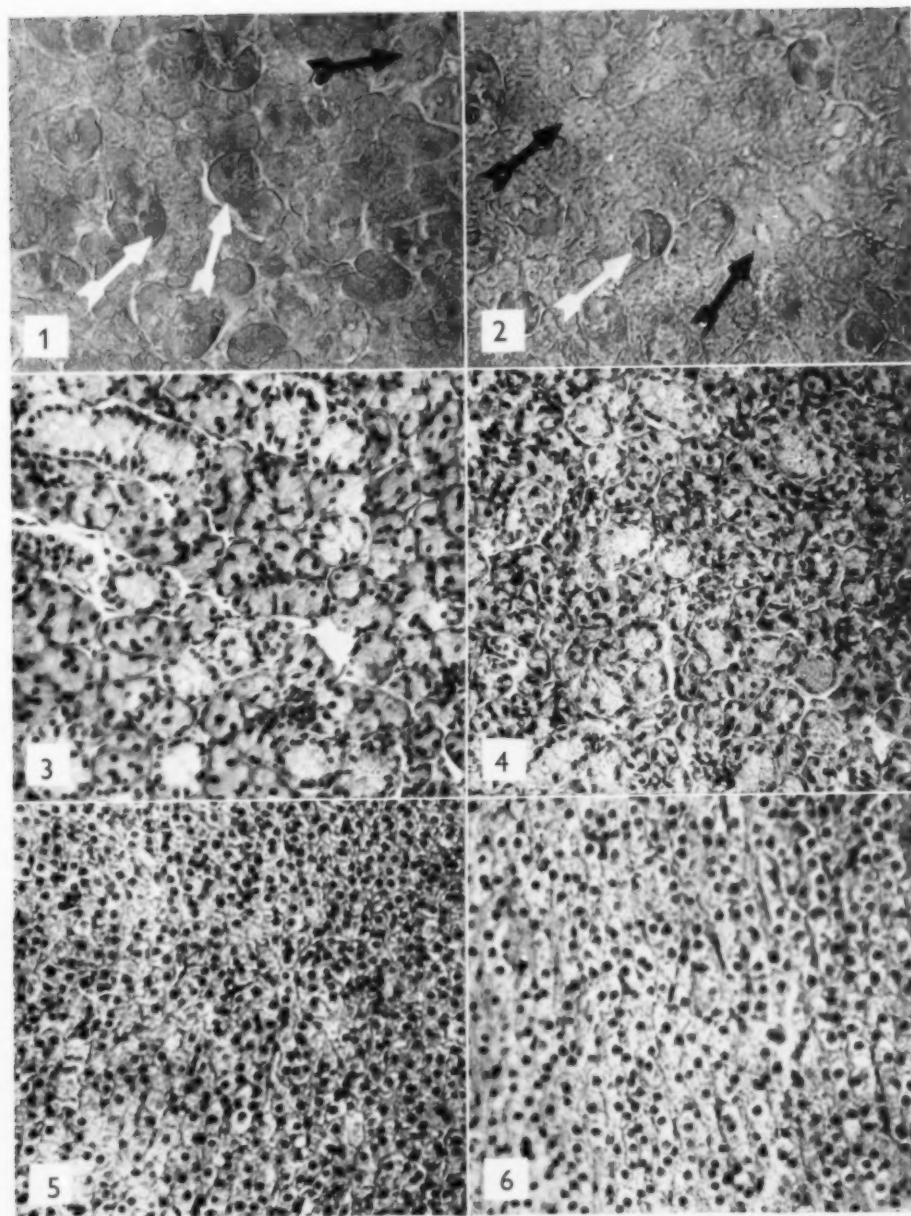
PLATE 1
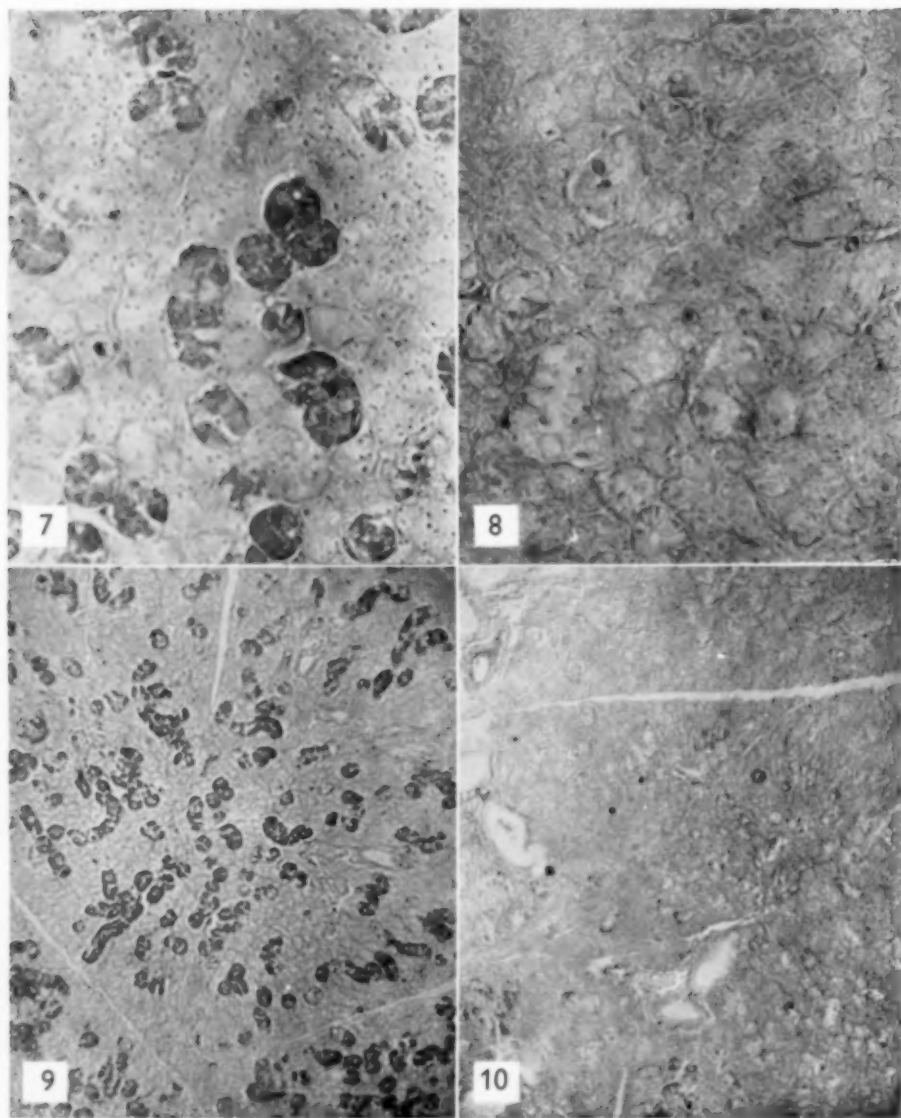
FIG. 7. The gland shown in Fig. 5, stained after 18 hr of digestion with malt diastase. Note the few positive ducts. Periodic acid–Schiff. ×40.

FIG. 8. The gland shown in Fig. 6 stained after 18 hr of diastase digestion. Almost all of the ducts stain PAS-positive. Periodic acid–Schiff. ×40.

FIG. 9. Higher magnification of part of the section shown in Fig. 7. Note the negative ducts. Periodic acid–Schiff. ×110.

FIG. 10. Higher magnification of part of the section shown in Fig. 8. Note the positive ducts. Periodic acid–Schiff. ×110.

PLATE 2

THE RELATIONSHIP BETWEEN SOME ANATOMICAL FEATURES OF THE HUMAN MANDIBULAR CONDYLE AND ITS APPEARANCE ON RADIOGRAPHS

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Abstract—With the aid of a Lindblom apparatus adapted to produce shadowgraphs, twenty-four condyles were examined for the following features: the angulation of the transverse axis of the condyle to the frontal and horizontal planes of the skull, and the degree of definition of the articular facets on the condyle. It was concluded that these features were not related to one another, and that they could not be determined from radiographs prepared by the Lindblom technique.

INTRODUCTION

IT has previously been shown (BERRY and CHICK, 1956) that if a controlled radiographic technique is used, differences in mandibular condyle shape seen on a series of radiographs of different condyles are caused by variations in their anatomical form. As a logical extension of that work, an investigation was made to determine (a) whether certain features of the anatomical form of the condyle are related to each other, and (b) whether these features could be determined by an inspection of a radiograph of the condyle obtained by a standard dental technique.

MATERIAL AND METHODS

The material studied was a series of twenty-four undamaged adult human skulls with complete dentitions, selected from the stock of a firm supplying osteological specimens.

(a) *Measurement of the angulation of the condyle axis to the frontal plane of the skull*

The mandible, detached from its skull, was photographed in such a position that the occlusal plane was parallel to the plane of the film. The resulting photograph was
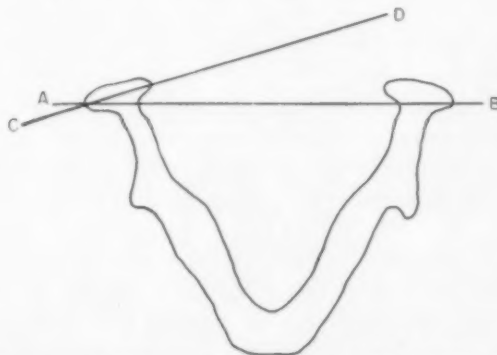
FIG. 1. Measurement of the angulation of the condylar axis to the frontal plane of the skull. CD represents the condylar axis. AB, joining the lateral poles of the condyles, is assumed to be parallel to the frontal plane.

placed face downwards on a brightly illuminated viewing-screen. The poles of the right condyle, i.e. those points on the medial and lateral sides of the condyle where the radius of curvature was smallest, were marked and joined by a line to represent the condylar axis (Fig. 1). The angle between this line and another joining the lateral poles of the right and left condyles was measured. This measurement represented the angulation of the condylar axis to the frontal plane of the skull. The angulations of the condylar axes of the twenty-four right condyles were recorded in degrees.

(b) *Measurement of the angulation of the condyle axis to the horizontal plane*

The mandible, detached from its skull, was photographed in such a position that the occlusal plane was at right angles to the plane of the film. The resulting photograph was placed face downwards on a brightly illuminated viewing-screen and the procedure described in (a) above was repeated. The angle between the condylar axis and the inter-condylar line joining the lateral poles of the condyles was measured (Fig. 2). The angle represented the inclination of the condyle axis in the horizontal plane. The angulations of the twenty-four right condyles were recorded in degrees.
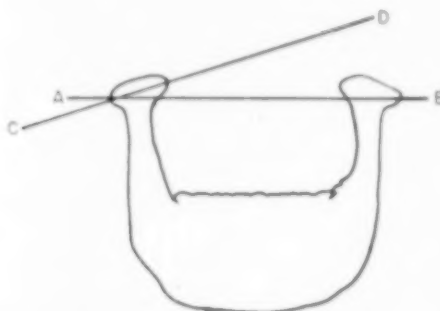
Fig. 2. Measurement of the angulation of the condylar axis to the horizontal plane. CD represents the condylar axis. AB, joining the lateral poles of the condyles, is assumed to be parallel to the horizontal plane.

(c) *Assessment of the degree of definition of the articular facets on the antero-superior surface of the condyle*

Examination of the twenty-four right condyles showed that the articular facets were well marked on some, poorly marked on others, and absent from the remainder. Thus three groups of condyles could be formed, each group having a characteristic degree of definition of the articular facets (Fig. 3).

(d) *The determination of certain features of the anatomical form of the condyle from a radiograph obtained by a standard dental technique*

Firstly, a record was required of the radiographic shape of the condyle assuming that the condyle axis orientation was subject to variation in both frontal and horizontal planes (i.e. a record of all the possible radiographic shapes which might be obtained from one condyle). In the earlier part of the investigation the variation of the angulation

of the condyle axis to the frontal plane was found to be from $+5°$ to $+33°$. Only two recordings in the series were above $25°$, and because the mean of the variation is $17°$, it was decided to record the radiographic shapes over a range of $0°$ to $25°$ angulation to the frontal plane.

The variation of the angulation of the condyle axis to the horizontal plane was found to be from $-21°$ to $+12°$. Only two recordings in this series were below $-15°$, and because the mean of the variation is $-3°$, it was decided to record the radiographic shapes over a range of $-15°$ to $+15°$ to this plane.

TABLE 1.   GRID-CHART SHOWING THE FORTY-TWO DIFFERENT POSITIONS IN
WHICH RECORDS OF THE CONDYLE WERE MADE

	$0°$	$5°$	$10°$	$15°$	$20°$	$25°$
$-15°$	1	8	15	22	29	36
$-10°$	2	9	16	23	30	37
$-5°$	3	10	17	24	31	38
$0°$	4	11	18	25	32	39
$+5°$	5	12	19	26	33	40
$+10°$	6	13	20	27	34	41
$+15°$	7	14	21	28	35	42

Angulation of the condylar axis to the frontal plane is set out across the grid. Angulation of this axis to the horizontal plane is shown vertically.

It was considered that a difference of $5°$ between recording positions would reveal any significant change in radiographic shape. Inspection of Table 1 shows that to achieve this difference between each recording, forty-two different records are required for each condyle.

The selected mandible was mounted on a "pan-and-tilt" device, similar to that used for photographic work, which permitted rotation in two planes, horizontal and transverse. The device was calibrated in degrees in both planes, and was controlled by fine-pitch screw adjusters. The mandible was mounted in such a way that the condylar axis was horizontal, and this axis was also aligned with a datum line marked on the bench to represent the frontal plane. A Lindblom apparatus was modified so that a light beam of the same dimensions as the normal dental X-ray beam produced a shadow of the condyle on a sand-blasted Perspex screen carried in the standard cassette-slide of the apparatus (Fig. 4). The mandible was then moved by the screw adjusters so that the condyle was in Position No. 1 on the grid-chart shown in Table 1. The shadow on the screen was photographed. The condyle was then placed, by means of

a screw adjuster, in each position on the chart in order of numbering, and a photo-graph made in each position. The series of forty-two shadowgraphs thus produced were then mounted in the same order as is shown in Table 1.

Secondly, a recording of the radiographic shape of the condyle was required when the condyle was regarded, for purpose of orientation, as being part of its own mandible, the latter being correctly positioned for the Lindblom technique.

These records may be abstracted from those obtained above.

RESULTS

The angulation of the condyle axes to the frontal plane and to the horizontal plane, and the degrees of definition of the articular facets are listed in Table 2. In obtaining the angulation measurements the assumption was made that the line joining the lateral poles of the condyles lies on planes parallel to the frontal and horizontal planes of the skull. This is open to criticism, but the exact orientation of these planes

TABLE 2

Skull no.	Condyle axis orientation		Condyle facets*	Skull no.	Condyle axis orientation		Condyle facets*
	To frontal plane (degrees)	To horizontal plane (degrees)			To frontal plane (degrees)	To horizontal plane (degrees)	
1	+7	+2	H	13	+28	+7	M
2	+12	−7	H	14	+13	−21	O
3	+25	−6	H	15	+14	−4	M
4	+21	−11	H	16	+21	+2	O
5	+18	−9	H	17	+21	−6	M
6	+17	−3	H	18	+19	+6	O
7	+8	−6	M	19	+18	+5	O
8	+12	−7	O	20	+23	+2	O
9	+14	−18	O	21	+33	+12	O
10	+22	−7	O	22	+12	+4	H
11	+19	+7	M	23	+13	0	O
12	+12	−6	O	24	+5	−10	M

* H=well marked; M=poorly marked; O=absent.

with respect to the skull, which is invariably asymmetric, is always arguable. It was considered that this procedure did not introduce any significant error.

For recording purposes the conventions adopted by CRADDOCK (1953) were used to express the "sense" of the angulation of the condyle axis. With respect to the frontal plane, when the lateral pole of the condyle is more anterior than the medial, the angulation of the condyle axis is given as a "positive" figure. With respect to the horizontal plane, when the lateral pole of the condyle is superior to the medial, the figure for the angulation is preceded by a "minus" sign: where the reverse is found, a "positive" sign is used.

(a) the variation in the angulation of the condylar axis to the frontal plane is from $+5°$ to $+33°$, the mean being $17°$ (S.D. $6·4$);

(b) the variation in the angulation of the condylar axis to the horizontal plane is from $-21°$ to $12°$, the mean being $-3°$ (S.D. $7·8$).

These figures may be compared with those given by CRADDOCK. Although the methods by which he obtained them are not described, there is sufficient similarity between the two sets of figures to claim that they are mutually confirmatory.

Inspection of Table 2 also shows that there is no simple relationship between:

(i) the angulation of the condylar axis to the frontal plane and its angulation to the horizontal plane (Cols. 2 and 3);

(ii) either of the above and the degree of definition of the articular facets (Col. 4).

It would therefore appear that there is no simple relationship present between the three anatomical features examined.

By using the modified Lindblom apparatus in the manner described, a series of forty-two shadowgraphs was obtained for each of the twenty-four right condyles. An example of a typical series is shown in Fig. 5.

By using the condylar axis angulations peculiar to each condyle as cross references, the shadowgraphs corresponding to the radiographic shape of the condyle obtained when the condyle was regarded as part of its own mandible was marked by a disc in the upper right hand corner (Fig. 5).

Thus each series of shadowgraphs showed both the theoretically possible radiographic shapes which might be obtained by radiographic examination of the condyles, and the actual shape obtained, if the Lindblom apparatus was used in the usual way.

An attempt was made to classify the twenty-four sheets of condyle shadowgraphs into groups, each group having similar characteristics. At the start of the attempt it

TABLE 3

Skull no.	Pattern of possible radiographic shapes (Groups A–H)	Shapes obtained when skull correctly orientated for Lindblom technique (Groups T–Z)	Skull no.	Pattern of possible radiographic shapes (Groups A–H)	Shapes obtained when skull correctly orientated for Lindblom technique (Groups T–Z)
1	E	V	13	B	W
2	C	Y	14	F	V
3	C	T	15	C	U
4	G	Y	16	C	Y
5	C	Y	17	C	X
6	C	Y	18	B	T
7	A	Y	19	B	Y
8	D	Y	20	D	X
9	F	V	21	A	U
10	D	V	22	C	W
11	H	Z	23	D	X
12	D	V	24	E	U

was at once realised that not even two of the twenty-four sheets were identical. But certain basic similarities could be found sufficiently frequently to permit the sheets to be placed in various groups with some consistency. Clearly this visual grouping depended on the observer's appreciation of shape and the degree of difference between groups considered significant. However, after twenty attempts, it was found that the same seven groups had been produced on twelve occasions. After twenty attempts certain shadowgraphs became memorized, and it was felt that the procedure became invalid. These seven groups are given in Table 3 (Col. 2).

A similar procedure was carried out, selecting only those shadowgraphs marked with a disc. The same observer determined eight groups with the same consistency as had been achieved with the grouping of the patterns of possible radiographic shapes. These eight groups are given in Table 3 (Col. 3). Inspection of Table 3 shows that there is no simple or consistent relationship between the two columns.

A further search for a relationship which might exist between any of the factors in both Tables 2 and 3 was made by tabulating each factor against all the others in turn, producing a further twenty tables. No relationship was found.

DISCUSSION

The failure to find any relationship between:
- (a) the selected anatomical features of the mandibular condyle;
- (b) the actual radiographic shape obtained by a standard technique and the theoretically possible radiographic shapes obtainable by this technique; or
- (c) any of the features in (a) and (b) above, emphasizes the limitations of radiography of the temporomandibular joint.

Previous work (BERRY and CHICK, 1956) has shown that the zones of accurate representation on the standard dental lateral–oblique radiographic projections of the joint are very narrow, and that only one of these zones is likely to be within the actual joint space. Further, that differences in the shape of the radiographic outline are due to differences in anatomy between joints. The evidence presented here suggests that it is impossible to deduce any definite information concerning certain features of the anatomy of the condyle from an inspection of its shape on a radiograph, and it may be concluded that the angulation of the condyle axis to the frontal plane and to the horizontal plane, and the degree of definition of the articular facets of the condyle, do not appear to be inter-related. Nor does it appear to be possible to obtain any information concerning these features from a radiograph of the temporomandibular joint made by the Lindblom technique.

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(a)   (b)   (c)
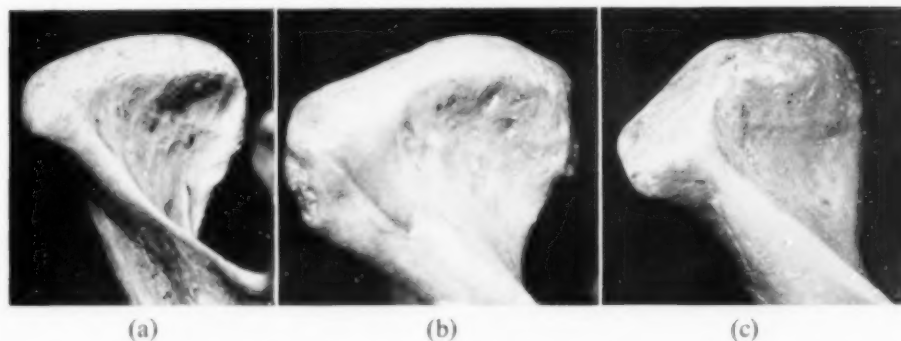
FIG. 3. Photographs of the anterior surfaces of the mandibular condyles, showing the degrees of definition of the anterior facets. (a) Absence of facets. (b) Poorly-marked facets. (c) Well-marked facets.
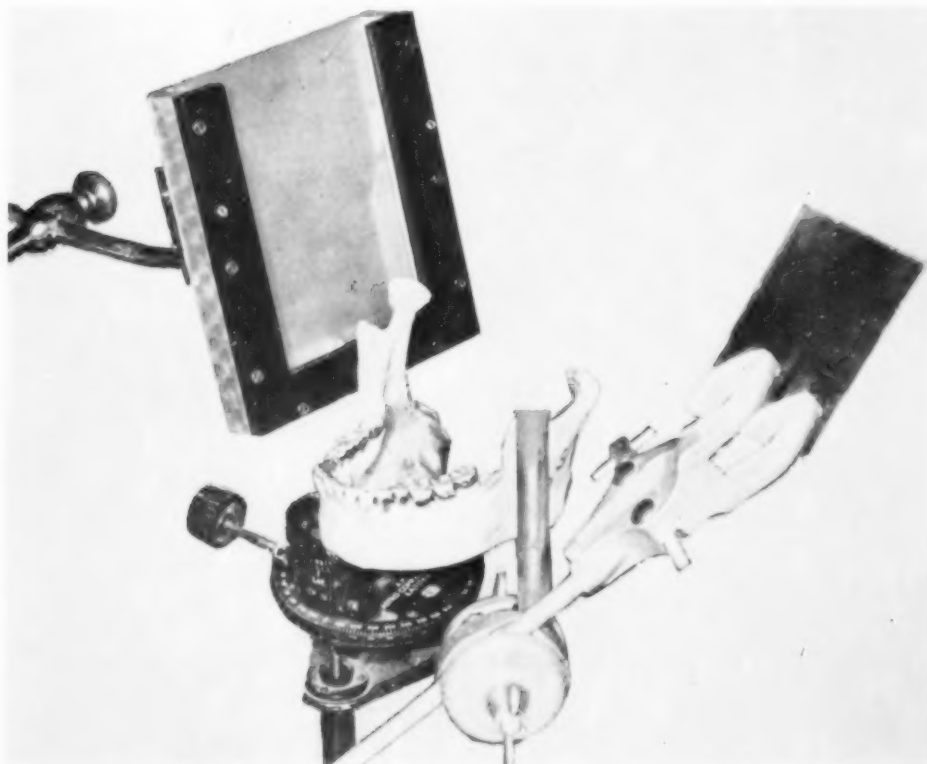
FIG. 4. The Lindblom apparatus modified for the production of shadowgraphs.

PLATE 1
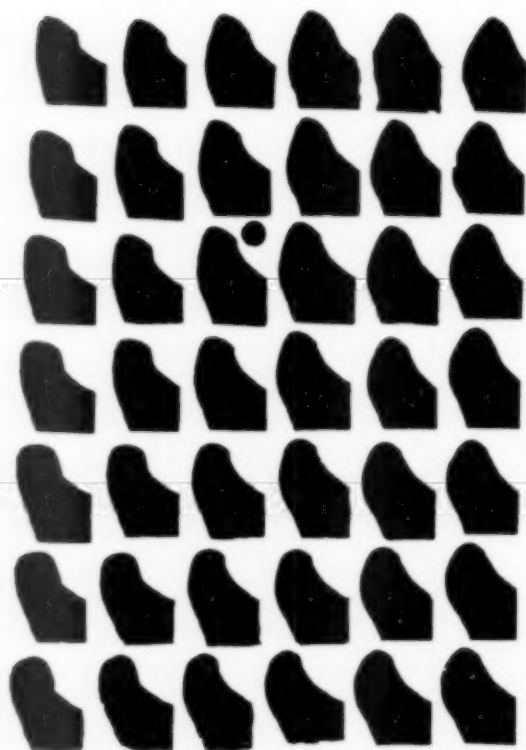
FIG. 5. A series of forty-two shadowgraphs of one condyle.

PLATE 2

THE INFLUENCE OF FAMILY SIZE ON THE PREVALENCE OF DENTAL CARIES IN CHILDREN

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Abstract—A comparative study of dental caries prevalence was made between 283 first-born children with no siblings and 830 children whose birth-rank order was third or later. These children comprised 149 "only" children and 500 of later birth rank in the age group 5-8 years, between whom comparison of caries experience of the deciduous teeth was made. For comparison of caries in the permanent teeth, 134 "only" children were compared with 280 children of later birth rank, with an approximate age of 13 years and who had erupted not less than twenty-five and not more than twenty-eight permanent teeth.

The results show that the average number of decayed, missing and filled teeth per child is lower in those whose birth rank was "third or later", compared with the "only" children. These findings are compared with those of two earlier studies in which the reverse relationship was found and the differences between this present study and the earlier studies are discussed.

The literature cited provides evidence to show that size of family exerts a measurable influence upon family economics, diet and nutrition. The differences in caries between the birth-rank orders are thought to be related to the direct effect of diet upon the oral environment of the tooth. This suggests, therefore, that susceptibility to caries can respond in a sensitive manner to relatively small changes in the dietary.

It is concluded that dietary differences due to social and economic conditions are sufficient to influence susceptibility to dental caries in school children.

THE influence of diet and nutrition upon the susceptibility to dental caries is well known. Moreover, standards of living, when they influence diet, may also be expected to influence the prevalence of this disease.

Family size can exert a considerable influence upon the economic circumstances of the family and, in families where income is fixed, an increase in size must inevitably result in an alteration in the standards of living in direct proportion to the size of the family. Changes in pattern of diet and a decline in nutritional level following increase in size of family have been shown (LORIMER and ROBACK, 1940; BELTRAM, 1949; NATIONAL FOOD SURVEY COMMITTEE, 1955). Furthermore, it has been observed that children from small families tend to be of better physique than those from large families, especially in the lower social groups (MINISTRY OF EDUCATION, 1954).

These data indicate clearly that in spite of the changes and improvements in social and economic conditions since the war, economic differences between different social levels still exist to a degree that can produce a reduced standard of nutrition with increase in size of family.

Differences in caries between children of differing rank orders of birth have been reported in Britain (MILLER and CROMBIE, 1939) and in the U.S.A. (BERK, 1943). However, the study by MILLER and CROMBIE was made in pre-World War II conditions

on a small number of children selected to provide the extremes of susceptibility to caries, that is, half the children were caries-free and half highly susceptible. On the other hand, the investigation of BERK was confined to 5-year old American children.

The present study was therefore undertaken to see if consistent differences in caries existed between children of differing rank order of birth under existing socio-economic conditions, and furthermore, if such differences existed, to see if they would be found in both the deciduous and permanent teeth.

METHODS

The data were obtained from two samples. The first consisted of 2304 children selected to provide a good cross-section of Social Classes III, IV and V (REGISTRAR-GENERAL, 1931); the second sample consisted of 1730 children selected to provide proportional representation of all social classes. The total number examined amounted therefore to 4034 children, i.e. 1972 boys and 2062 girls, aged 5–17 years.

Because social class differences in the prevalence of caries had been found in these children (MANSBRIDGE, 1960), children attending fee-paying schools were excluded from this study.

Birth rank

For each child, the rank order of birth was recorded. This information was obtained from the older children by direct question, but for the younger children the class teacher obtained this information.

For comparison of the effects of family size on the prevalence of caries, two groups of children were selected, thus:

 (i) those who had no siblings, and

(ii) those whose rank order of birth was third or later.

These two birth-rank groups therefore permitted the comparison between children from the smallest possible family and those who had not less than two other siblings. This seemed the most appropriate comparison in view of the finding that for those families with three or more children, the diet was lower than generally recommended for protein, calcium and possibly riboflavin (NATIONAL FOOD SURVEY COMMITTEE, 1955).

From the two different birth-rank groups there were selected:

(a) all children from 5 to 8 years of age, i.e. 364 boys and 355 girls, and

(b) all children who had erupted not less than twenty-five and not more than twenty-eight permanent teeth, i.e. 177 boys and 237 girls.

The first group permitted a comparison of dental caries of deciduous teeth between children of differing birth rank, while the second enabled a similar comparison to be made for the permanent teeth.

Dental examination

The children were examined in the schools during school hours and the examinations were made using plane mouth mirrors and Ash No. 54 probes. Illumination was obtained by the use throughout of a portable lamp using a 60 W bulb.

Measurement of dental caries

The D.M.F. index (KLEIN, PALMER and KNUTSON, 1938) has been used to measure the prevalence of dental caries.

RESULTS

Deciduous teeth

It was found that when the children were ranked in order of birth, the average number of decayed, missing and filled (D.M.F.) deciduous molar and canine teeth per child decreased from the "only" children to those whose rank order was third or later. The summarized data are presented in Table 1.

TABLE 1.   DECIDUOUS TEETH. THE MEAN NUMBER OF DECAYED, MISSING AND FILLED (D.M.F.) DECIDUOUS MOLAR AND CANINE TEETH IN CHILDREN AGED 5–8 YEARS, RANKED IN THEIR ORDER OF BIRTH

Birth rank	Boys ($t = 2.8$; $p < 0.01$)				Girls ($t = 2.1$; $p < 0.05$)			
	No. of children	Mean D.M.F. teeth per child	Standard deviation (D.M.F.)	Mean age per child (yrs)	No. of children	Mean D.M.F. teeth per child	Standard deviation (D.M.F.)	Mean age per child (yrs)
Only child	65	7·01	3·03	6·8	84	6·89	3·23	6·8
Third or later	299	5·80	3·51	7·2	251	6·02	3·47	7·3
Total	364	6·01	3·46	7·1	335	6·24	3·43	7·2

From Table 1 it can be seen that the average number of decayed, missing and filled teeth is substantially less for those children whose birth rank is third or later than it is for those who are "only" children. The differences in the mean number of D.M.F. teeth between the two different birth ranks were found to be statistically significant at the 1 per cent level of probability for the boys and at the 5 per cent level for the girls.

Permanent teeth

The findings in respect of the permanent teeth are summarized in Table 2.

It can be seen that for both sexes the average number of decayed, missing and filled teeth per child is less for those whose rank order of birth is third or later than it is for the "only" children. Thus, the same trend in the prevalence of caries was found in the permanent teeth of the older children as was found in the deciduous teeth of the younger group. The differences in the average number of D.M.F. permanent teeth between the different birth ranks were found to be statistically significant for the girls at the 1 per cent level of probability, but not statistically significant for the boys.

There was no logical reason to expect substantial differences in age between the birth ranks and, in the children in whom the deciduous teeth were studied, it was found that differences in age between the birth ranks did not correspond with the

TABLE 2. PERMANENT TEETH. THE MEAN NUMBER OF DECAYED, MISSING AND FILLED (D.M.F.) PERMANENT TEETH IN CHILDREN WHO HAD ERUPTED NOT LESS THAN TWENTY-FIVE AND NOT MORE THAN TWENTY-EIGHT PERMANENT TEETH, RANKED IN THEIR ORDER OF BIRTH

Birth rank	Boys ($t=1\cdot2$; $p<0\cdot3$)				Girls ($t=2\cdot8$; $p<0\cdot01$)			
	No. of children	Mean D.M.F. teeth per child	Standard deviation (D.M.F.)	Mean age per child (yrs)	No. of children	Mean D.M.F. teeth per child	Standard deviation (D.M.F.)	Mean age per child (yrs)
Only child	43	6·23	4·15	14·0	91	6·97	3·30	13·9
Third or later	134	5·39	3·14	13·7	146	5·76	3·05	13·4
Total	177	5·59	3·42	13·8	237	6·22	3·19	13·6

differences in caries (see Table 1). For this reason and also because of the similarity in trend in the prevalence of caries in the deciduous and permanent teeth with change in birth rank, it was thought that the differences in age between the birth ranks of the older group contributed little, if at all, to the differences in caries between them.

DISCUSSION

The results obtained in this study show that, when the children were grouped according to their rank order of birth, the average number of decayed, missing and filled teeth declined from the "only" child to those whose birth rank was "third or later". This finding was common to both deciduous and permanent teeth and to both sexes and, together with the statistical anylysis, suggests that these findings are un-likely to have arisen by chance.

Differences in the prevalence of caries in British children due to differing social and economic backgrounds have been reported (WILKINS, 1941; COUMOULOS and MELLANBY, 1947; MANSBRIDGE, 1960). Therefore, in seeking an explanation of the present findings, the most reasonable explanation would seem to be found in the differences in diet which can occur between families of different sizes due to economic causes.

The Annual Report of the National Food Survey Committee for 1953 shows that, in Britain, increase in size of family may exert a measurable influence upon family economics and diet. Thus it was found that for all items of diet there was a progres-sive reduction in amount with each increase in size of family. Moreover, for those families with three or more children, the diet was lower than recommended for protein, calcium and possibly riboflavin. It was suggested that these are the nutrients in which the larger families are likely to be most vulnerable during a period of rising prices.

BELTRAM (1949) found that children of unskilled workers and children of families of four or more children tended to eat more of foods such as bread, stews and meat puddings and less of cakes, biscuits, cheese, eggs and fruit, than children of skilled

workers and "only" children. In the United States, LORIMER and ROBACK (1940) found that, as the number of children increased, families at given income levels compromised between increased food requirements and other added needs by accepting a more restricted diet.

These various findings clearly show that increasing family size in certain social groups leads to a less lavish or even to a poorer diet. Furthermore, there is ample evidence to show that in individuals who subsist on a more restricted diet, the incidence of dental caries is generally low. In institutional children a low level of caries is a frequent finding.

It is also possible that with increase in size of family, the characteristics of institutional life are to some extent reproduced on a smaller scale. Thus, not only does the diet become plainer, but pandering to individual dietary preferences of the children becomes less likely.

The findings relating to birth rank and the prevalence of dental caries in Edinburgh children are in distinct contrast with those of MILLER and CROMBIE (1939) and BERK (1943), both of whom found that the later born children of a family had a higher level of caries than the older children. There are, however, important differences between the two previous studies and the present study.

Firstly, the numbers of children are smaller in both the earlier studies than they are for the present one and secondly, the children examined both by MILLER and CROMBIE and by BERK were derived from lower socio-economic groups. The Boston children studied by BERK came from families who could not afford private dental treatment, yet were sufficiently enlightened to appreciate the need for it.

The study of MILLER and CROMBIE was carried out in Tyneside on children from a very low socio-economic group, many of whom were born and reared during a period of economic depression and unemployment, which was very marked in that area.

ORR (1937) showed that for the same period of time in Britain that, in the lower income groups, the average diet was considered inadequate for perfect health and that, in fact, a diet completely adequate for health by modern standards was reached only at an income level above that of 50 per cent of the population. Furthermore, he commented that, owing to their nutritional needs during growth and development, the effects of poor diet were accentuated in the children. In these circumstances, increase in family size could only depress further the nutritional level of the family and consequently each succeeding child would tend to be reared in even poorer circumstances than those of his immediate predecessor.

The differences in dental caries between the younger and older children in a family may, under such social conditions, reflect the effects of poor pre-natal nutrition and sub-optimal standards of nutrition of the infant and child during the period of tooth development with consequent inferior tooth structure. However, with regard to diet following tooth eruption, it is possible that, although nutritionally poor, it contained less of the caries-provoking substances found in the diets of the more prosperous members of the community. Consequently, the differences in post-eruptive environment of the teeth between the first and later born children would not have differed so greatly in terms of caries-producing factors.

It seems possible, therefore, that the findings of MILLER and CROMBIE, and BERK, may be explained in terms of these dietary variations. The disparity in the findings between the two previous studies and the present one seem, therefore, to be due to two main causes. Firstly, the effects of school milk and meals at nominal cost, welfare foods, full employment and high wages have all contributed to raise the standards of living well above the pre-war level. Therefore, differences in nutrition between the various birth ranks in this study were probably insufficient to result in very marked differences in tooth structure. Secondly, the Edinburgh children were derived from Social Classes III, IV and V (REGISTRAR-GENERAL, 1931) and therefore probably cover a wider socio-economic spectrum than existed in the earlier studies.

It is suggested, therefore, that the differences in caries between the differing ranks of birth in the Edinburgh children studied would seem most likely to be a reflection of changes in the post-eruptive oral environment due to the economics of family size, each increase in family size reducing the level of consumption of those foods which may be regarded as luxuries, but affecting least those items of diet which are essential to health.

It is concluded, therefore, that differences in diet resulting from social and economic conditions can exert an influence upon the susceptibility to dental caries in school children that is by no means negligible.

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RADIOISOTOPES IN THE TEETH OF DOGS—I

THE DISTRIBUTION OF PLUTONIUM, RADIUM, RADIOTHORIUM, MESOTHORIUM AND STRONTIUM AND THE SEQUENCE OF HISTOPATHOLOGIC CHANGES IN TEETH CONTAINING PLUTONIUM

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Abstract—Jaws and teeth containing radioisotopes from adult Beagles were studied to determine the location of the activity and the sequence of histopathologic changes. Radioautography after a single intravenous injection of bone-seeking radioisotopes (plutonium, radium, mesothorium, radiothorium and strontium) in young adult Beagles yielded the same distribution pattern in dental tissues. The sequence of changes observed in dogs injected with plutonium (serially sacrificed dogs and dogs allowed to live until moribund) involved the formation of secondary dentine, disturbance in cementum formation, resorption of cementum and dentine with metaplastic bone formation, damage to periodontal membrane, ankylosis of teeth and loss of teeth. There was a direct relationship between the sites of plutonium deposits and locations of dental lesions. No tumour arose from dental tissue, but a few osteogenic sarcomas occurred in the jaws.

INTRODUCTION

THE following observations are part of a larger study of the comparative toxicities of plutonium-239, radium-226, thorium-228 (RdTh), radium-228 (MsTh) and strontium-90 in Beagles. It is known that these radioisotopes deposit principally in mineralized tissues and will cause radiation dysplasia and neoplasia (VAUGHAN, 1956; FINKEL, 1956; OWEN, SISSONS and VAUGHAN, 1957). Although it has been over thirty years since MARTLAND (1929) showed that gingivitis, buccal infection, necrosis and osteomyelitis of the jaw are among the first symptoms to appear in radium toxicity, there has been only one histologic study of teeth in rabbits fed radium reported to date. The limited availability of data on this subject may be due to the fact that earlier investigators have assumed that the uptake and distribution of the isotopes in teeth would be similar to bone and have neglected to study the distribution of radioisotopes and the histopathologic changes in dental tissues.

The present paper describes the sites of deposition of radioisotopes in the teeth and jaws of dogs injected with a single intravenous dose of plutonium, radium, radiothorium, mesothorium or strontium using the autoradiographic method. Also autoradiography is combined with routine histologic and radiographic methods to determine the location of plutonium-239, in the entire post-exposure period and the subsequent development of dental and jaw lesions including malignant tumours.

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MATERIALS AND METHODS

The materials involved in these studies are the dental tissues and jaws from six normal Beagles and forty-two others that died or were sacrificed from 1 to 1724 days following the administration of radium-226, plutonium-239, radium-228, thorium-228 and strontium-90 (Table 1). These animals are part of the Utah Beagle chronic toxicity studies of bone-seeking radioelements at the Radiobiology Laboratory of the University of Utah College of Medicine. The physical characteristics of these parent radioisotopes and the total radiation energies of the decay products are listed in Table 2. A complete summary of the complex chain of daughter products of these parent isotopes can be found elsewhere (AUB, EVANS, HEMPELMAN and

TABLE 1. SUMMARY OF EXPERIMENTAL DOGS

Parent isotope	Dose (μc/kg)	No. of animals
Plutonium-239	2·43–3·30	20
Plutonium-239	0·81–1·03	6
Plutonium-239	0·26–0·29	3
Radium-226	9·68–10·8	5
Radium-226	3·51	1
Thorium-228 (RdTh)	0·88	2
Radium-228 (MsTh)*	9·93–10·1	3
Strontium-90	93·6–111·0	2
	Controls	5
	Total	47

* Contaminated with 3% radiothorium-228

TABLE 2. PHYSICAL CHARACTERISTICS OF THE BONE-SEEKING RADIOELEMENTS

Parent isotopes	Primary emission (MeV)	Half life (yr)	Radiation energies (decay products) (MeV)			Major deposition sites
			α	β	γ	
Plutonium-239	5·23 α	24,300	5·23	—	—	Mineralized tissues & liver
Radium-226	4·77 α	1620	29·24	1·15	1.93	Mineralized tissues
Radium-228 (MsTh)	0·1 β	6·7	31·26	1·13	3·04	Mineralized tissues
Thorium-228 (RdTh)	5·40 α	1·9	31·26	0·71	2·08	Mineralized tissues & RE system
Strontium-90	0·20 β	28·0	—	1·13	—	Mineralized tissues

TABLE 3

Exp. no.	Age at injection (months)	Injected dose ($\mu c/kg$)	Burden time (days)	Cause of death	Accum. dose rads	No. of teeth missing
Plutonium						
T0P5	21	3·05	1	Sacrificed	6	0
T15P5	24	2·79	1	Sacrificed	5	0
T13P5	19	3·16	3	Sacrificed	20	0
T14P5	19	2·43	7	Sacrificed	30	0
T9P5	19	2·80	15	Sacrificed	80	0
T10P5	18	2·74	15	Sacrificed	70	0
T11P5	17	2·76	28	Sacrificed	140	0
T12P5	16	2·74	28	Sacrificed	140	0
T16P5	22	2·85	92	Sacrificed	440	0
T17P5	24	3·01	210	Sacrificed	990	0
T18P5	24	2.83	217	Sacrificed	960	0
T5P5	16	2·69	400	Sacrificed	1720	1
T6P5	17	2·73	406	Sacrificed	1750	0
M3P5	17	3·00	499	Hepatitis	2340	0
T7P5	16	2·68	777	Sacrificed	3230	0
T8P5	17	2·67	863	Sacrificed	3540	2
F6P5	13	2·57	1194	Osteosarcoma	4620	25
M1P5	14	2·67	1324	Osteosarcoma	5370	12
M4P5	19	3·17	1562	Osteosarcoma	7380	23
F2P5	38	3·30	1576	Osteosarcoma	7830	29
M4P4	19	0·974	1066	Osteosarcoma	1560	4
M3P4	16	0·929	1198	Osteosarcoma	1680	14
F5P4	21	0·872	1245	Osteosarcoma	1650	10
F6P4	14	0·811	1357	Osteosarcoma	1660	13
F2P4	19	1·03	1556	Osteosarcoma	2380	26
M1P4	15	0·823	1724	Osteosarcoma	2100	17
M1P3	14	0·261	1476	Osteosarcoma	580	2
F5P3	21	0·288	1504	Osteosarcoma	630	12
M3P3	16	0·291	1604	Osteosarcoma	700	8
Radium						
T16R5	20	9·68	12	Sacrificed	230	0
M2R5	15	10·8	1380	Osteosarcoma	15,500	16
M5R5	15	10·1	1220	Osteosarcoma	13,120	29
M7R5	15	11·9	1288	Osteosarcoma	16,200	12
F9R5	14	9·16	1288	Osteosarcoma	12,900	31
M1R4	15	3·51	1606	Osteosarcoma	5790	21
Radiothorium						
T6T4	19	0·884	651	Osteitis	3570	5
M4T4	20	0·886	793	Osteitis	4110	8
Mesothorium and 3% Radiothorium						
T3M5	19	10·1	1115	Anaemia	18,400	4
F2M5	15	10·0	780	Osteitis	11,200	0
M3M5	19	9·93	689	Anaemia	9430	3
Strontium						
F1S5	14	93·6	960	Osteosarcoma	7860	1
M2S5	15	111·0	255	Hernia	3440	0
Controls						
M35A			529	Sacrificed		0
F01A			1380	Sacrificed		4
M03A			1431	Sacrificed		0
M36A			1971	Sacrificed		0
F10A			2605	Sacrificed		0

MARTLAND, 1952; MAYS, STOVER, GLAD and ATHERTON, 1959). The experimental dog code, age at injection, injection dose, burden time, cause of death, accumulative irradiation dose and number of missing teeth are listed in Table 3. Four symbols are used to identify a dog; the first identifies the sex ("M" for male or "F" for female), the second (1–12) designates the injection group, the third the injected radioelement ("P" for Pu^{239}, "R" for Ra^{226}, "M" for Ra^{228}, "T" for Th^{228}, "A" for aging control), and the fourth (1–5) for the dose levels. Thus, M1P5 is a male dog in the first group of dogs and is injected with the 5-level dose (3·0 μc/kg) of plutonium and F10A is a normal female aging control.

Augmenting the chronic toxicity study are tissues from fifteen plutonium dogs and one radium dog which were serially sacrificed to give information regarding distribution of the radioelements and the sequence of histopathologic changes of the mineralized tissues during the first 2 yr following injection. These animals are labelled with a "T" instead of "M" or "F".

The experiment was designed so that young adult Beagles of mixed sexes aged between 14 and 24 months were given a single intravenous injection of one of the various dose levels of bone-seeking radioelements. The injection solution of radio-isotopes was prepared in a citric acid–sodium citrate buffer, 0·08 M in total citrate and pH 3·5 (VAN DILLA et al., 1958; STOVER, ATHERTON and KELLER, 1959; STOVER et al., 1957; STOVER and ATHERTON, 1958). The animals were permitted to live until moribund, when they were sacrificed with sodium pentobarbital and examined for gross and miscroscopic evidence of disease. The care, housing, serial radiographs and clinical histories of these dogs will be reported elsewhere (REHFELD, personal communication). At autopsy, the entire skeleton was studied and some of the findings have been reported elsewhere (JEE, OTTOSEN, MICAL and LOWE, 1957; JEE et al., 1959; ARNOLD and JEE, 1959). All but a few of these animals had bone tumours at the time of death (Table 3). For this study, the jaws were disarticulated and the surrounding soft tissues removed for postmortem radiographs. Sawn segments of the mandible bearing the premolar teeth and of the maxilla bearing the canine and premolar teeth were fixed in Zenker–formol for histologic study. The mandible with the first and second molars were fixed in acetone for autoradiographic studies and the remainder of the jaws and teeth were stored in acetone.

Histologic technique. The specimens fixed in Zenker–formol for 24 hr were decalcified in 6% sulphosalicyclic acid, trimmed in blocks and dehydrated in alcohols and ether–alcohol, infiltrated in 3 and 6% Parlodion (a purified pyroxylin made by Mallinkrodt Chemical Works, St. Louis, Missouri) for 5 and 10 days respectively. The infiltrated blocks were embedded in 12% Parlodion, cut at 15μ and stained with Hansen–Bock haematoxylin and eosin.

Contact autoradiography. The specimens fixed in absolute acetone for 48 hr were dehydrated and defatted in several changes of ether–alcohol and absolute ether and embedded in plastic (Selectron 5003. Manufactured by Pittsburgh Plate Glass Co., Pittsburgh, Pennsylvania). Each plastic block containing the teeth and mandible was sawn into 500 μ thick labiolingual sections by a "Di-met" cutter with a diamond saw blade (Model 11R; Felker Mfg. Co., Torrance, California). The sections were ground

down to 100 μ in thickness between two glass plates using levigated alumina as the abrasive suspended in absolute alcohol. The sections were polished in alcohol between the same glass plates. At no time did the sections come in contact with any aqueous medium, which may leach the radioelements. The polished sections were sandwiched between two pieces of 25 μ thick, Ilford C-2, nuclear research emulsion coated on 5/1000 in. acetate base (Ilford Ltd., Ilford, Essex, England). The two pieces of nuclear emulsion and the section were in turn sandwiched between two pieces of $1\frac{1}{2}$ in. thick foam rubber in a plexiglass press and clamped together by bolts and wing-nuts. The plexiglass press is similar to the apparatus described by LOTZ, GALLIMORE and BOYD (1952). The press with the nuclear emulsions was stored in a light-tight can in the deep freeze ($-20°C$) for exposure times ranging from 18 to 90 days. The nuclear emulsions were developed in Kodak developer D-19, fixed in Kodak acid fixer, rinsed in running tap water, dried and mounted on microslides.

OBSERVATIONS

The observations have been divided into several portions. The first section deals with autoradiographic studies of the distribution of plutonium-239, thorium-228 (RdTh), radium-226, radium-228 (MsTh) and strontium-90 in the dental tissues and their supporting structures. The remaining sections (entitled Radiographic and Histopathologic Studies) deal only with those observations made on the dogs given three different dose levels of plutonium-239.

Autoradiographic Studies

It is surprising to observe that the localization of the five bone-seeking radio-elements in teeth is quite similar, even though the microscopic distribution of plutonium and radiothorium in bone differs from that of radium, mesothorium and strontium (OWEN, SISSONS and VAUGHAN, 1957; ARNOLD, 1954; JEE et al., 1958).

Localization of plutonium and radiothorium. As reported in a preliminary study, the location of plutonium and radiothorium in teeth and supportive structures is quite similar (ARNOLD, 1955). For brevity, only three autoradiographs of labio-lingual sections of the first molar and mandible at 1, 777 and 1576 days after the administration of plutonium are presented to exemplify some of the representative localization patterns. At day 1, the chief sites of blackening in the autoradiograph may be summarized as follows (Fig. 1):

(1) An intense concentration demarcating the newly formed dentinal surface of the pulp chambers;

(2) A moderate deposit of activity lining the vascular canals in the apical portion of the root;

(3) A much lighter amount on the surface of the cementum and the alveolar bone enveloping the periodontal ligament;

(4) A drastically reduced deposit of activity on the enamel surface; and

(5) A blackening lining the various bone surfaces of the mandible, such as the bone surfaces of the endosteum, periosteum, Haversian canals, trabeculae and resorption cavities.

At 777 days after the administration of plutonium, the isotope is deposited diffusely in the dentine formed after the injection of the isotope (post-injection dentine) in addition to the sites observed at day 1 (Fig. 2). The post-injection dentine formed initially incorporates very little, but this is followed by dentine showing a heavier uptake of activity. The low uptake of plutonium adjacent to the initial intense line is due to the brief exposure of rapidly apposed dentine to circulating plutonium in the blood in the period following the acquisition of the isotope. The more recent post-injection dentine formed near death is dentine forming at a much slower rate, thus allowing more time for the activity in the blood to be incorporated in the newly formed dentine. Also at this time, we can detect autoradiographically the recession of the alveolar crests and the translocation of the activity in bone as the result of remodelling. These changes in bone have been reported elsewhere (JEE *et al.*, 1957; JEE *et al.*, 1959) and will not be described here.

The 1576 days post-injection autoradiogram exhibits post-injection dentine with a more intense labelling of plutonium; but again, we note the same general distribution of activity (Fig. 3). The more recently formed dentine incorporates more activity. We can also detect the loss of activity due to the resorption of cementum, alveolar crests and alveolar bone.

Localization of radium. The localization of radium in teeth is similar to the deposition pattern of plutonium and radiothorium. It differs only in the diffuse penetration of radium into a small portion of the dentine (Fig. 4). In the mandible, the radium is found to be deposited in intense concentrations called "hot spots" in newly formed bone (regions of appositional growth) and diffusely throughout the remainder of bone existing at the time of the administration of radium. Unlike the plutonium and radiothorium deposition patterns where the highest concentration is found only in the initial dentinal surface, the more intense concentrations of radium are found in the newly formed dentine, cementum and bone. The post-injection dentine is labelled with a uniform deposit of radium, which approximates the concentration observed in the diffuse component of bone. This differs markedly from the varying concentration in post-injection dentine in teeth containing plutonium and radiothorium. A very light but observable localization of radium is restricted to the surface of the enamel and does not penetrate into the enamel proper.

Localization of mesothorium. The distribution of mesothorium is quite similar to the localization of radium and differs only in the very light enamel surface deposition of activity (Fig. 5).

Localization of strontium-90. Strontium-90, a beta-emitter, differs from the radium deposition pattern only in its slight diffuse penetration of the enamel (Fig. 6).

Radiographic Studies

The radiographic study will only deal with the findings from the animals injected with 3·0, 0·9 and 0·27 μc/kg of plutonium. Due to the limitation in space, only a selection of eight radiographs of the disarticulated and flesh-free mandibles with teeth are shown to illustrate some of the representative changes. Similar alterations also appear in the upper jaws and in animals receiving various doses of radium,

mesothorium and radiothorium. The changes induced by the latter three alpha-emitting, bone-seeking isotopes will be described in detail in a future report.

Normal teeth and mandible. The dental formula for the lower jaw of a Beagle is three incisors, one canine, four premolars and three molars. Fig. 7 shows the mandible with the full complement of eleven teeth from a 17 month-old control dog. The critical sites where changes occur are the delicate interdental trabeculae, periodontal ligament, pulp chambers and alveolar crests normally extending up to the cervical region of the teeth. Fig. 8 shows a mandible from a 48 month-old Beagle, illustrating some of the normal age changes in teeth and their supportive structures. There are obvious alterations in that the interdental trabeculation becomes coarsened and disorganized, the width of the periodontal ligament is narrowed or often obliterated and the alveolar crests have receded from the necks of the teeth. Frequently a normal dog in this colony will have a few teeth missing (Table 3).

Animals injected with 3·0 μc/kg *of plutonium.* Unequivocal changes in the mandible and teeth were observed in this group of dogs after 800 days. Fig. 9 shows a mandible from a Beagle sacrificed 863 days after injection. The trabeculation is smudged and disorganized. The coronal portions of the pulp chambers, especially of the premolar teeth, appear to be filled with calcified material while the periodontal ligament is narrowed or obliterated and the alveolar crests have receded. However, this mandible still contains the normal complement of eleven teeth.

Very advanced changes are observed in two dogs sacrificed 1180 and 1562 days post-injection (Figs. 10 and 11). There is severe resorption of teeth, which is found to occur most frequently at the necks of the teeth. The alveolar crests have receded to such an extent that only half of the roots are embedded in the mandibular bone. Many teeth are missing and the remaining teeth exhibit loss of the periodontal ligaments and are held in place by fusion with the interdental trabeculae and the alveolar bone (ankylosis). The coronal portion of the pulp cavities of the teeth is often filled with calcified material and the entire mandible is "moth eaten" in appearance (zones of rarefaction).

Extraordinary destruction is seen in the jaw of a dog surviving 1576 days post-injection in which only three incisors, one canine and one molar remain fixed to the mandible (Fig. 11). The body of the mandible exhibits zones of extreme rarefaction and the region posterior to the first molar has been completely resorbed and is held together by soft tissue. Histologic examination showed this to be an osteogenic sarcoma.

Animals injected with 0·9 μc/kg *of plutonium.* Figs. 12 and 13 illustrate the typical changes observed in the teeth and mandible in this injection group. These two dogs succumbed 1198 and 1556 days after the administration of plutonium with changes that are similar to, but less extensive than those described for the dogs injected with 3·0 μc/kg.

Animals injected with 0·27 μc/kg *of plutonium.* The condition of the teeth and jaws of this group was quite variable. Fig. 14 shows a representative mandible of this group with minimal resorption of the teeth, some loss of periodontal ligament and teeth, coarsening of trabeculation and receding alveolar crests. Only occasionally

are there zones of rarefaction in the mandible. The extent of the damage is less pronounced than the preceding two groups; however, the periodontal tissues are unequivocally more deteriorated than the control animal of comparable age (Fig. 8).

Histopathologic Studies

The histologic examination of teeth and their supportive structures containing plutonium was carried out principally at the lower premolars, upper canine and molars.

Normal. The histologic appearance of the dental and periodontal structures of the Beagle are identical to those of man. Fig. 15 shows the normal appearance of a labiolingual section of a premolar and mandible from a 17 month-old animal, while Figs. 21, 25 and 31 show higher magnifications of the relationships of the alveolar bone, periodontal ligament, cementoblasts, cementoid layer, cellular cementum and dentine. The age changes of the teeth of dogs are also identical with those in man. There is a continual slow apposition of dentine and cementum with consequent reduction in the size of the pulp cavity and the width of the periodontal ligament while simultaneously the alveolar crests recede toward the apical parts of the roots. Frequently secondary dentine formation as well as loss of periodontal ligament and bone–tooth union (ankylosis) are seen in the controls (Fig. 16).

The sites and timing of changes in teeth of dogs injected with 3 μc/kg of plutonium. There is a direct relationship between the sites of primary damage and the localization of the plutonium. We described earlier the intense concentration on the dentinal surface lining the pulp cavities, moderate localization on the mineralized surfaces of the root canals, cementum and the alveolar bone and all bone surfaces of the mandible exposed to a vascular supply. Correspondingly, histopathologic changes have been observed in the pulp chambers (odontoblasts), necks and roots of teeth involving the cementum and dentine, periodontal ligament and alveolar bone.

Pulp cavity changes. The earliest change is seen in the odontoblasts lining the coronal tip of the pulp chambers in premolars and the narrow communicating isthmus between coronal pulp chambers in molars. The geometry of these sites causes an approximate doubling of the radiation dose received by their lining odontoblasts, as compared to odontoblasts which line the remainder of the pulp cavity; there is an abnormal proliferation of secondary dentine occurring as early as 3 months post-injection (Fig. 18). In the molar teeth, the secondary dentine proliferation obliterates the communicating passage between the two lobes of the pulp chambers (Fig. 20). The remaining odontoblasts lining the pulp cavities appear to be unaffected by the intense deposition of activity (Fig. 18). Frequently, there is an inconsistent basophilic staining of the post-injection dentine, which may be due to disturbed dentine calcification. Microradiographic studies are planned to confirm this interpretation.

Delayed changes (2½ yr) are observed in the pulp cavities, involving the laying down of secondary dentine in response to the massive resorption of cementum and dentine (Fig. 35). The secondary dentine often obliterates the chambers (Fig. 33). Another late change is the plugging of many root canals, which results in the diminution of the vascular supply to the pulp cavity.

Root changes. Although the surfaces of both the acellular and cellular cementum are coated with approximately equal amounts of activity, the former shows pathologic changes involving a shorter latent period than the cellular cementum. Nevertheless, identical sequences of changes are observed at both types of cementum. There is disturbed cementoblastic activity resulting in the reduction of cementoblasts (Fig. 22), loss of the cementoid layer (Fig. 23), apposition of abnormal basophilic-staining cementum and the formation of pericemental fibrosis (Figs. 24 and 26). This is followed by punctate areas of resorption of cementum and adjacent dentine involving osteoclasts in Howship's lacunae (Fig. 27). Disturbed cementoblastic activity on the acellular cementum occurs as early as 3 months and is followed by resorption at 7 months after the acquisition of plutonium, whilst the identical events in respect of the cellular cementum occur at 7 and 13 months respectively. The disturbed cellular cementum also shows a localized distribution of empty lacunae in the post-injection cementum (Fig. 26). The distinct basophilic cemental borders and pericemental fibrosis are seen at 400 days (Fig. 26), followed by severe resorption of cementum and dentine at 777 days post-injection.

Late changes involve the substitution of cementum, lamellar bone or gingiva for dentine in the affected areas (Figs. 28, 33, 34, 35). Generally the apposition of lamellar bone or cementum forms a union of mineralized tissues (ankylosis; Figs. 28, 29, 33, 35). There is often severe resorption at the cervical region of the tooth resulting in sloughing of the crown, which leaves behind the root ankylosed to the mandible (Fig. 29).

Periodontal ligament. The periodontal ligament, surrounded by sheaths of plutonium deposited on the surfaces of the cementum and alveolar bone, succumbs to the late effects of the irradiation. The first detectable change is observed in the disturbance in vascularity of the ligament proper, occurring at approximately 1 yr post-injection. Large dilated vessels appear where formerly vessels of normal calibre were seen (Figs. 31 and 32). This is followed by the loss of organization and function of the ligament at $2\frac{1}{2}$ yr. Sharpey's fibres are no longer present to anchor the tooth to the tooth socket, but in their place are fibres running parallel to the cementum and alveolar bone surfaces (Fig. 32). These teeth are usually lost if no bone–tooth union occurs as described below (Figs. 28, 29, 33, 35). The loss of teeth is severe at 3 yr post-injection (Table 3).

Alveolar bone. The bone constituting the tooth socket is much more reactive to the damaging effects of the irradiation than the dental tissue. The first signs of abnormal tissue change is the progressive reduction of osteoblasts lining the alveolar bone surface, resulting at 3 months post-injection in the loss of osteoblasts and the osteoid layer, and resorption of the alveolar crest. Many osteoclasts in Howship's lacunae are observed at sites of bone resorption. At 6 months the basal bone of the alveolus is resorbed. This is followed by a proliferation of new bone resulting in a union of the bone with dentine or cementum (Figs. 28, 29, 33, 35). The ankylosed tooth has been observed as early as 400 days post-injection. The frequency and severity of root resorption and ankylosis increase as the function of burden time. Much later ($2\frac{1}{2}$ yr) when the teeth are falling out, there is massive resorption of necrotic

alveolar bone and apposition of atypical bone at many sites to compensate for the severe osteolysis (Fig. 30).

Mandibular bone. Changes in the trabecular and cortical bone constituting the mandible are identical with those observed in other areas of trabecular and cortical bone in the body. These changes have been described in detail elsewhere (JEE *et al.*, 1957; JEE *et al.*, 1959). In summary, there are early changes (as early as 3 months) which include peritrabecular and endosteal fibrosis, bone resorption, localized bone necrosis and the apposition of atypical bone. Late changes involving principally the cortical bone include the formation of Haversian canal plugs, resulting in bone necrosis, followed by disturbances in bone remodelling. These result in the formation of abnormally numerous and abnormally shaped Haversian systems, eroded peri-osteal surfaces with fibrosis, spontaneous fractures and osteogenic sarcomas. The bone tumours arise predominately from the regions of spongy bone.

Occurrence of tumours in jaws. Although in this study all but a few of the animals receiving plutonium had one or more osteogenic sarcomas at the time of death, only one animal developed a tumour in the jaw. So far, no tumour has arisen from the dental tissues. One animal given 3·3 μc/kg of plutonium has a lesion which was primarily diagnosed radiographically as a fracture, but proved to be an osteogenic sarcoma on histologic examination (Fig. 11).

Comparative histopathologic studies. The histopathologic changes in teeth in controls and dogs given 3·0, 0·9 and 0·27 μc/kg of plutonium are listed in Table 4. The changes listed in Table 4 have been described in detail for the animals injected with 3·0 μc/kg. There is surprisingly little difference in the degree of damage between animals injected with 3·0 and 0·9 μc/kg of plutonium, even though there is a three-fold spread between the two injected doses. The main differences are more extensive bone and tooth resorption, more tooth loss and less Haversian bone in animals receiving the highest dose. The animals receiving 3·0 μc/kg of plutonium had an

TABLE 4. HISTOPATHOLOGIC FINDINGS OF UNTREATED TEETH AND TEETH CONTAINING PLUTONIUM-239

Dose Level (μc/kg) No. of dogs Burden time (months)	3·0 4 39–52	0·9 7 35–47	0·27 3 49–53	0 5 17–86
Odontoblast death	0	0	0	0
Secondary or reparative dentine	+	+	±	±
Cementocyte death	+++	++	±	0
Alveolar crest resorption	+++	+++	+	±
Alveolar bone resorption	+++	++	+	0
Non-functional periodontal ligament	+++	+++	0	0
Loss of periodontal ligament	+++	++	0	±
Eroded dentine and cementum	+++	+++	+	0
Bone-cementum or dentine union (ankylosis)	+++	+++	±	±
Haversian bone in dentine	+	+++	+	0
Tooth loss	+++	++	+	±

average tooth loss of twenty-four, ranging between twelve and twenty-nine teeth, while the 0·9 μc/kg group averaged eleven teeth, ranging from four to twenty-six teeth. A thorough study of the tooth loss of the entire chronic toxicity programme has been reported elsewhere (REHFELD, FISHER and NIELSEN, 1958).

In the 0·27 μc/kg injected group, the changes are markedly less. Occasionally there are secondary or reparative dentine, cementum and alveolar bone resorption, Haversian bone in dentine and fusion of bone and tooth. Frequently, the teeth appear as healthy as in the controls of comparable age. The loss of teeth averaged seven and ranged between two and twelve teeth in this group.

The control animals were not free from the changes listed for the toxicity animals. In general, the teeth were not resorbed and changes only involved secondary dentine formation, periodontal ligament injury, alveolar bone resorption (Fig. 16) and tooth loss. In the few normal animals studied, only one had lost four teeth (Table 3).

DISCUSSION

It is apparent from the present and earlier investigations that, in dental tissues, bone-seeking radioisotopes are localized in a few sites owing to the restricted blood supply (AUB *et al.*, 1952; LOONEY, 1951; MCLEAN, CALHOUN and AUB, 1954; HOLGATE, 1959; ENGSTROM, BJORNERSTEDT and CLEMEDSON, 1957; MYERS, 1955; WAINWRIGHT, 1951). The isotopes are found lining the surfaces of cementum, dentine, enamel and alveolar bone, whilst the bulk of the dentine and enamel is free of activity. We found that the localization of alkaline earths (radium, mesothorium and strontium) differs from the localization of actinide radioelements (plutonium and radiothorium) in that the former penetrate a short distance into the dentine, while the latter are restricted to the dentine surface. The uptake of alkaline earth radioisotopes in teeth differs drastically from that of bone in the absence of activity in old dentine, while old bone is completely labelled by heterionic exchange (ARNOLD, 1954). Due to the restricted distribution of these isotopes, the concentration of activity in whole teeth has been found to be less than bone (MCLEAN, CALHOUN and AUB, 1954; EDINGTON, WARD, JUDD and MOLE, 1956; HOLGATE, MOLE and VAUGHAN, 1958).

As in bone, the concentration of alkaline earth radioisotopes in zones of active calcification in teeth appears approximately to equal that of bone ("hot spots"), while the surface concentration of plutonium on newly formed dentine is equal to the highest concentration on bone surfaces (TWENTE and JEE, 1958).

In general, there appears to be little change in the distribution of the bone-seeking radioisotopes after the initial deposition except for the uptake of activity in post-injection dentine and alveolar bone. Our observations show that dentine formed rapidly immediately after the initial intense plutonium deposition incorporates less activity than more recent slowly formed dentine, even though there is more activity circulating in plasma when the former is laid down (STOVER, ATHERTON and KELLER, 1959). There is a possibility that an acceleration in the rate of dentine apposition occurring immediately after the acquisition of plutonium can decrease the incorporation time for the plutonium on dentinal surfaces. A more extensive autoradiographic study which would include the measurement of rates of dentine apposition should be

performed before this point can be clarified. However, it can be stated now that the dentine in question is histologically normal and should not be confused with secondary dentine, which incorporates very little activity due to its rapid rate of apposition (unpublished observation).

Once there is fixation of radioisotopes in permanent teeth, the removal of activity occurs only when teeth are lost by extraction or pathologic resorption. This permanent fixation of isotopes is not the same in animals whose teeth grow from persistent pulps, where the initial intense uptake of strontium-90 in areas of active calcification in pre-injection dentine is lost through attrition and replaced by growth of new post-injection dentine and enamel containing a much lower concentration of activity (HOLGATE, 1959; ENGSTROM, BJORNERSTEDT and CLEMEDSON, 1957). This differs also from the fixation of radioisotopes in bone, where the activity can be lost through remodelling (ARNOLD and JEE, 1959) and long term exchange (ROWLAND and MARSHALL, 1959).

In adult animals, the sites in teeth containing activity act as permanent local sources of irradiation. In the case of the alpha-emitters with their short range ($\sim 32\,\mu$), only the odontoblasts and a few adjacent capillaries are exposed to this intense activity. However, the cellular constituents are exposed only for a span of time until the continued apposition of post-injection dentine, containing a much smaller quantity of activity, provides a protective shield for the odontoblasts against the intense source of irradiation. With strontium-90, a beta-emitter with a greater range (6 mm in bone), penetration and bombardment of both the odontoblasts and vascular supply in the pulp cavities may occur, and so this isotope might be considered a potentially greater hazard to teeth than the short range alpha-particle emitters. However, due to the limited distribution of the strontium-90, much of its activity is also absorbed by the acellular dentine.

Certain features of the damage to teeth and their supportive structures should be discussed further. The radioresistance of odontoblasts, the pathogenesis of the unusual dental lesions, the occurrence of osteogenic sarcoma in jaws and the relationship between irradiation dose and injury will be considered.

In turning our attention to the dental lesions, it is interesting to note that, in the dose range used in this study, there is little or no direct irradiation injury to the odontoblasts in the pulp cavity except for an early localized secondary dentine proliferation. The odontoblasts are functionally analogous to the osteoblasts of bone and should be comparable in radiosensitivity; nevertheless, odontoblasts are found to be much more radioresistant. The odontoblast differs only in being a permanent cell in contrast to the transitory nature of the osteoblasts.

Dentine differs from bone in having a thick (10–$20\,\mu$) layer of non-calcified predentine which separates the plutonium from the odontoblasts but is not sufficiently thick to protect the odontoblasts from the $32\,\mu$ range of the plutonium alpha-rays lined along the dentine surface. In a preliminary report, the dentine concentration was estimated by quantitative autoradiography to be about five times higher than the heaviest plutonium concentration deposited on trabecular surfaces (TWENTE and JEE, 1958). The odontoblasts absorbing this intense irradiation are radioresistant,

while the osteoblasts lining the trabecular surfaces, and receiving much less activity, are destroyed (JEE *et al.*, 1957). Several other studies also have noted the high radioresistance of odontoblasts. A study in this laboratory, involving the measurement of the width of the pulp cavities in dental radiographs of Beagles injected with approximately 3 μc/kg of plutonium and controls of comparable age, showed no significant difference in width between these two groups (REHFELD, TAYLOR, FISHER and NEBEKER, 1957). Another study in which external irradiation in the range of 6900 r was used showed that the mature odontoblasts in adult dogs are "some of the most highly radioresistant cells of the body" (KALNINS, 1954).

The early abnormal response of odontoblasts laying down secondary or reparative dentine in restricted sites must not be ignored. The phenomenon has occurred in untreated animals from this colony in response to injury of dentinal tubules from attrition or caries. However, our study shows the response occurs soon after the administration of plutonium in teeth free from exposure of dentinal tubules to the above factors. The sites of secondary dentine proliferation are localized consistently at the so-called "coffin corners" or coronal tips of pulp cavities and the narrow communicating isthmi between coronal pulp cavities in molars where the odontoblasts may be receiving two to three times the amount of irradiation absorbed by the remaining odontoblasts. An earlier experiment by ROSENTHAL (1937), dealing with the rabbits fed orally about 50 μg/kg of radium sulphate, reported both the deposition of secondary dentine and disturbed dentine calcification. He does not state whether the secondary dentine proliferation is the result of the irradiation or is secondary to massive resorption of dental tissue.

The development of the unusual dental lesions described in this study appears to be the consequence of the direct effect of the irradiation upon the various cellular constituents of alveolar bone and cementum. For instance, we have described the suppression of cementoblastic activity resulting in the loss of cementoblasts and the proliferation of abnormal cementum. To date we have only studied the morphologic changes in cementum which include an abnormal staining reaction of the organic matrix and the loss of cementoid tissue. Cementum covered by a protective layer of cementoid tissue is accepted to be highly resistant to resorption (ORBAN, 1957). Also the localized foci of cementocyte death (devitalization) in areas of post-injection cellular cementum again suggest the effect of direct irradiation injury.

Following the apposition of defective cementum and bone without a continuous border of cementoid and osteoid tissue, resorption of bone, cementum and dentine occurs. The resorption sites appear to be identical with the unique dental decay at the necks and roots of teeth from radium patients (AUB, *et al.* 1952; LOONEY, HASTERLIK, BRUES and SKERMONT, 1955; LOONEY, 1956), patients receiving radiotherapy for oral cancer (DEL REGATO, 1939) and rabbits fed radium (ROSENTHAL, 1937) and injected with strontium-90 (HOLGATE, 1959). It has been suggested that resorption in the roots of teeth occurs when cementum becomes devitalized. EULER and MEYER (1927) demonstrate perforating resorption with extensive cavitation in reimplanted teeth in dogs, while GOTTLIEB and ORBAN (1931) pointed out that resorption occurs when the cementum of the root has lost its vitality. It is also a possibility that the

reimplanted tooth with a devitalized root is similar to a dead bone implant, for it has not only dead cementum but also lacks a protective non-calcified cementoid layer making it very susceptible to resorption.

DEL REGATO (1939) noted in patients receiving radiotherapy for cancer of the oral cavity that the resorption of the necks of teeth occurs outside the field of irradiation. He states that this indirect action can be explained by the action of the rays on the regional vascular system or from the irradiation of salivary glands and subsequent modification in the secretion of saliva. In the present study neither factor is relevant. No large regional blood vessels are within the range of the plutonium alpha-particles and autoradiographic studies show that the salivary glands picked up only a minute amount of plutonium (JEE, unpublished observation). However, no study on the chemical state of the saliva has been performed.

Subsequent to resorption the dentine is replaced by cementum, lamellar bone or gingiva. This has been described by oral pathologists as "idiopathic resorption with metaplasia" (THOMA, 1954). It also has been reported in the histopathologic study of teeth from rabbits fed radium sulphate (ROSENTHAL, 1937) and from radiographic study of radium patients (AUB et al., 1952). One group noted from dental radiographs that the dental lesions result from resorption from within the pulp (internal resorption, MARTLAND, 1929), while ROSENTHAL (1937) and our study show histologically that the resorption is initiated from without. An earlier investigation of "idiopathic resorption" supports the view of internal resorption but more recent studies involving serial histologic sections show resorption occurs from without. There also appears to be in the literature no histologic evidence of lacunar resorption originating from the pulp cavity (THOMA, 1954).

Although injury to blood vessels has been regarded as a typical feature of irradiation lesions (ELLINGER, 1957; RHOADES, 1948; CASARETT, 1952; ROWLAND and MARSHALL, 1959; ROWLAND, MARSHALL and JOWSEY, 1959; JEE and ARNOLD, 1959a) and circulatory disturbances in bones has been observed in these same animals (JEE et al., 1959; JEE and ARNOLD, 1959), we only observed dilated vessels in the periodontal ligament after 406 days post-injection. It is difficult to speculate if there is some connexion between the dilated vessels, vascular disturbance and destruction of the periodontal, dental and bone tissues. One isolated observation involving a dog burdened with plutonium for 406 days, whose circulatory system was accidentally injected with India ink, shows an unequivocal reduction of blood vessels with ink in the periodontal ligament. It may be that the late disturbance in vascular supply (400–800 days) resulting in the devitalization of bone and cementum enhances the early direct irradiation injury to dental tissue, which results in the massive resorption of bone, cementum and dentine seen after 1500 days.

It is not surprising to see osteogenic sarcoma arising from jaws in the animals injected with the bone-seeking radioisotopes since bone neoplasia is directly responsible for the death of over 90 per cent of our animals. The animals succumbing to causes other than bone tumours often had histologically detectable tumours. Many previous investigators have reported bone tumours arising in the jaws of humans with radium poisoning (AUB et al., 1952; LOONEY, 1956) and in animals given bone-seeking

radioisotopes (LISCO, FINKEL and BRUES, 1947; KOLETSKY, BONTE and FRIEDELL, 1950; FINKEL and BISKIS, 1959; GATES, 1943).

Besides the osteogenic sarcomas arising in one animal receiving plutonium, only three bone tumours have occurred in jaws in the forty-two animals listed in Table 3. Two were detected microscopically in the mandible of dogs injected with 10 μc/kg of radium (Fig. 37), while one was observed radiographically in the maxilla of a dog injected with 3·5 μc/kg of radium. All are osteogenic sarcomas, but not of the classical type. Histologically, the periphery of the tumours was very fibrous with centrally located strands of osteoid. It is not within the scope of this report to discuss the probable pathogenesis of the osteogenic sarcoma for this will be discussed in a future publication.

It is apparent that the histopathologic changes in teeth decrease in severity with diminishing injected doses (Table 4). With this in mind, an attempt can be made to correlate the accumulative dose in rads to the mineralized tissue with the degree of damage. The values of average skeletal radiation exposure in rads are obtained from the calculations in a report by MAYS et al. (1959), which takes into account the isotope retention, but not the microscopic distribution and particle range of plutonium (Table 3). The calculations assume a uniform distribution of the isotopes which have been shown in this and other studies to be invalid. At present data are being collected using microdensitometric scanning of autoradiographs of suitable material, which will take into account these factors in relating microdosimetry to dental lesions. In lieu of the more relevant values, it is proposed to use the average skeletal radiation exposure in Table 3, which will make it possible to compare the accumulative dose in rads between the three groups of plutonium dogs. Thus, the average terminal accumulative dose to the skeleton in rads in the group injected with 3·0 μc/kg range between 4650 and 7830 rads, the second group injected with 0·9 μc/kg range between 1590 and 1660 rads, while the third injected with 0·29 μc/kg range between 577 and 702 rads. It is apparent that the higher the accumulative dose, the greater the degree of damage (Table 4). However, there is some discrepancy in the strict association of dental lesions with the retained amount of radiation. For example, when comparing the radiation dose delivered to dental lesions in serially sacrificed dogs receiving 3·0 μc/kg and toxicity dogs injected with 0·27 μc/kg of plutonium, there is a wide spectrum of dental lesions in the latter group receiving 577–702 rads in 1600 days, while the former animal receiving 2000 rads in 800 days exhibited very few of the changes observed in the 0·27 μc/kg group. A probable explanation of the poor expression of dental lesions in the highest dose group may be that excessive irradiation may inhibit or delay the expression of dental lesions by the destruction of potential odontolytic, osteolytic, osteogenic and vascular forming cells. Other factors such as age, diet and infection should not be overlooked.

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PLATE 1

FIG. 1.   Deposition pattern of plutonium in tooth and mandible 1 day after injection.
×3·6.

FIG. 2.   Deposition pattern of plutonium in tooth and mandible 777 days after injection.   Note non-uniform deposits of activity in post-injection dentine (arrow).   ×3·6.

FIG. 3.   Deposition pattern of plutonium in tooth and mandible 1576 days after injection.   Note altered pattern.   ×3·6.

Fig. 4.   Deposition pattern of radium in tooth and mandible 12 days after injection.
×3·6.

FIG. 5.   Deposition pattern of mesothorium in tooth and mandible 780 days after injection.   ×3·6.

Fig. 6.   Deposition pattern of strontium-90 in tooth and mandible 960 days after injection.   ×3·6.
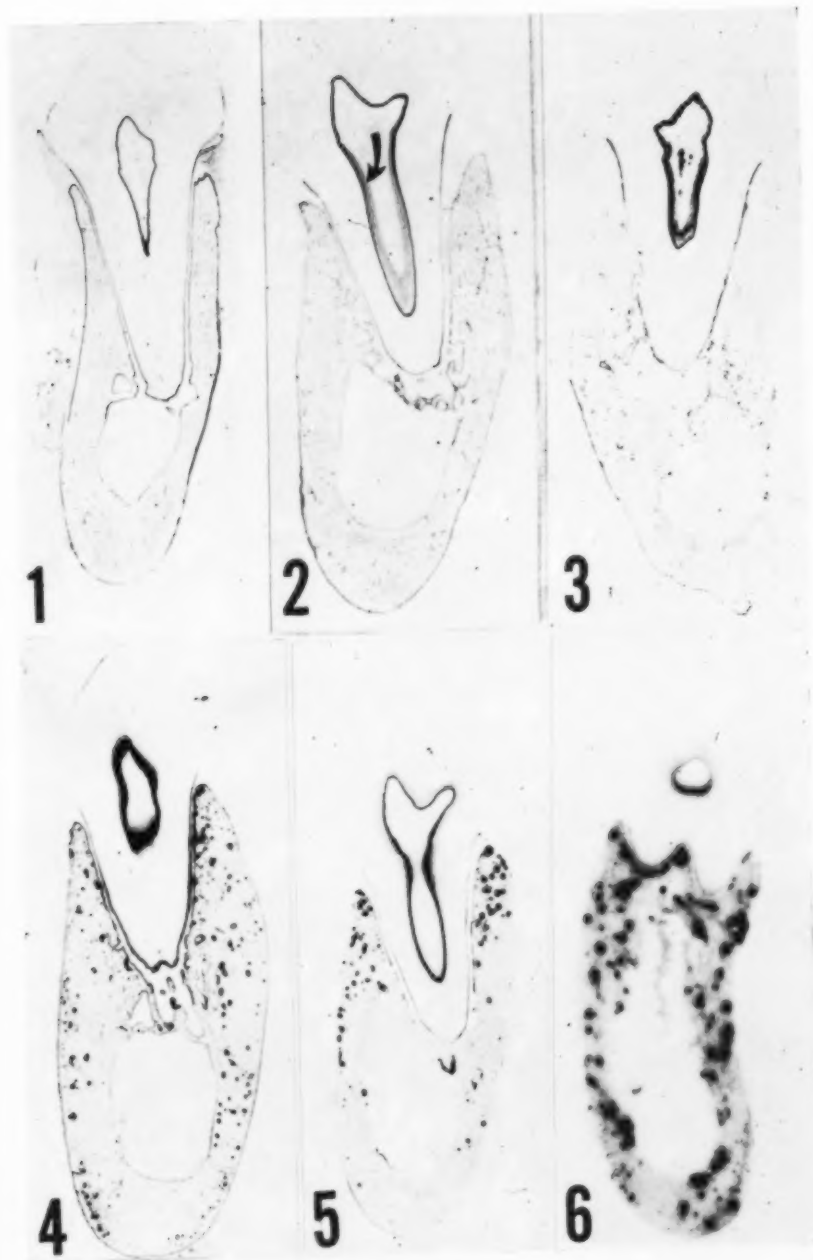
PLATE 1

Plate 2

Plate 2

Figs. 7–10 are postmortem radiographs of disarticulated, defleshed right mandibles from control and treated Beagles.

Fig. 7. Mandible of a 17 month-old control (M35A) showing the normal appearance of the interdental trabeculae (1), the periodontal ligament space (2) and the pulp cavity (3). × 0·9.

Fig. 8. Mandible of a 48 month-old control (M03A) demonstrating the changes with age. ×0·9.

Fig. 9. Mandible of a Beagle (T8P5) sacrificed 863 days after injection of 3·0 μc/kg of plutonium showing particularly the coarsened and smudged interdental trabeculae. ×0·9.

Fig. 10. Mandible of a Beagle (M4P5) sacrificed 1562 days after injection of 3·0 μc/kg of plutonium demonstrating the typical damage to the teeth and jaw at this dose level. Note especially the massive resorption of the roots of teeth and mandible. ×0·9.

PLATE 3

FIGS. 11–14 are postmortem radiographs of disarticulated, defleshed right mandibles from treated Beagles.

FIG. 11. Mandible of a Beagle (F2P5) sacrificed 1576 days after injection of 3·0 μc/kg of plutonium demonstrating the extreme destruction and loss of teeth. The area posterior to molar proved histologically to be an osteogenic sarcoma (arrow). ×0·9.

FIG. 12. Mandible of a Beagle (M3P4) sacrificed 1198 days after injection of 0·9 μc/kg of plutonium showing the typical damage to teeth and jaw at this dose level. ×0·9.

FIG. 13. Mandible of a Beagle (F2P4) sacrificed 1556 days after injection of 1 μc/kg of plutonium showing the most severe damage observed at this dose level. ×0·9.

FIG. 14. Mandible of a Beagle (M3P3) sacrificed 1604 days after injection showing much less damage than at the two higher dose levels. ×0·9.
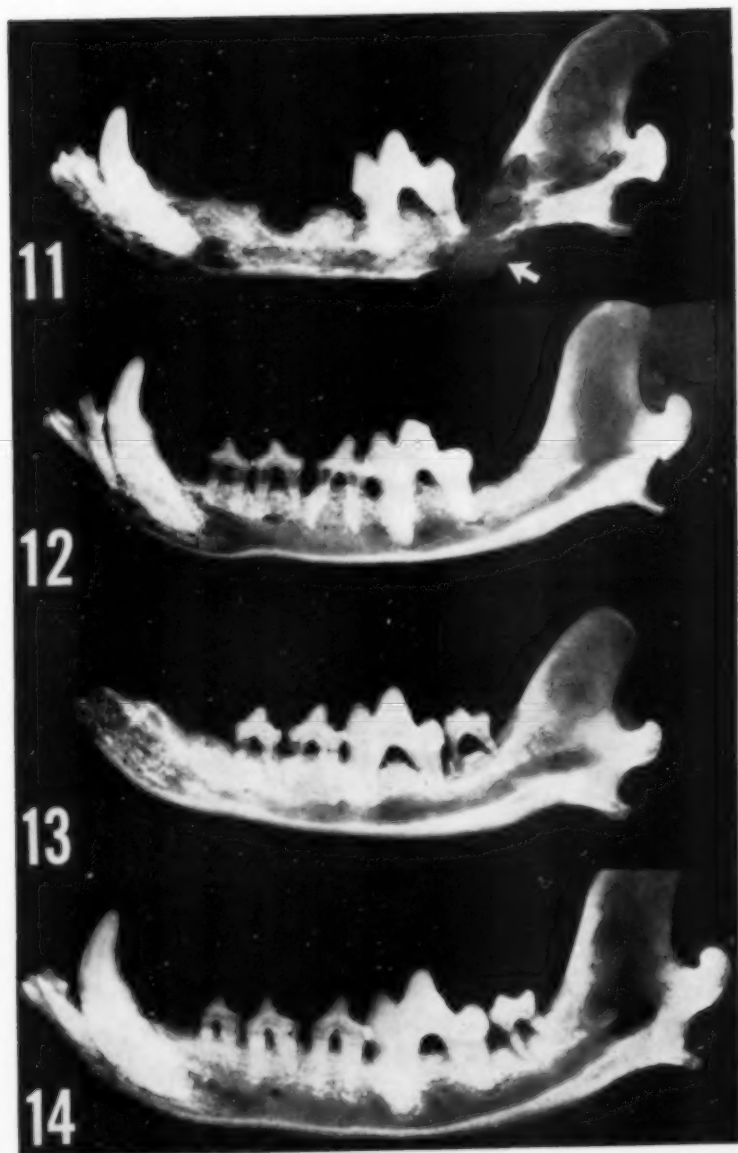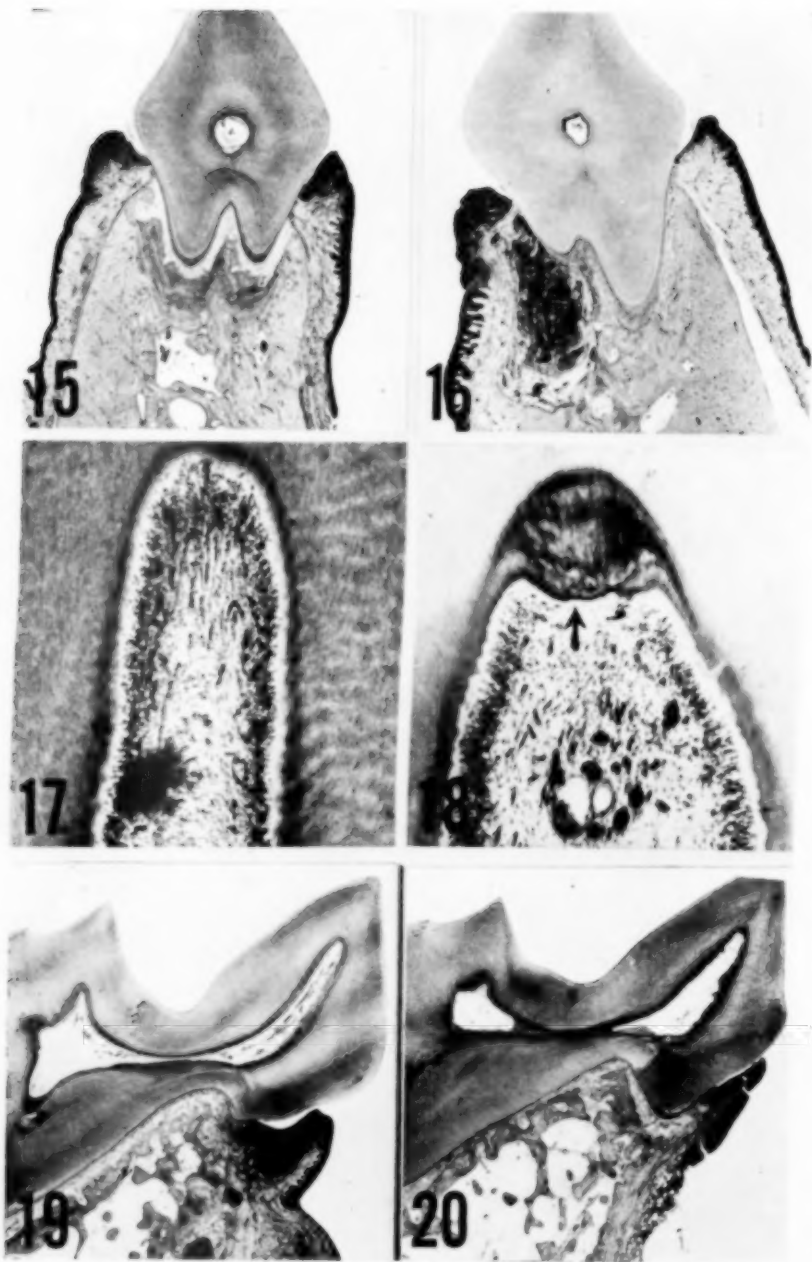
PLATE 3

Plate 4

PLATE 4

Fig. 15. Labiolingual section of premolar and mandible from 17 month-old control (M35A) showing the normal appearance of premolar and supporting tissues. ×9.

Fig. 16. Labiolingual section of premolar and mandible from 48 month-old control (M03A) demonstrating both age and pathologic changes. Note the thinned periodontal ligament, fused interdental trabeculae, and loss of one alveolar crest as the result of inflammation. ×9.

Fig. 17. Normal appearance of pulp cavity of a 48 month-old control (M03A). ×75.

Fig. 18. Pulp cavity of a premolar from a dog (T16P5) sacrificed 92 days after injection showing the occurrence of secondary dentine at the coronal tip. ×75.

Fig. 19. Normal appearance of coronal pulp cavities and connecting isthmus in a 48 month-old control (M03A). ×4·2.

Fig. 20. Plugged isthmus between pulp cavities in a Beagle (M4P5) injected with 3·0 μc/kg of plutonium. ×4·2.

Plate 5

Fig. 21. Normal appearance of dentine (D), acellular cementum (C), cementoid tissue (O) and two rows of cementoblasts in a 17 month-old control (M35A). ×600.

Fig. 22. Marked reduction in the number of cementoblasts 92 days after injection of 3·0 μc/kg of plutonium (T16P5). ×600.

Fig. 23. Reduced number of cementoblasts and loss of cementoid layer 210 days after injection of 3·0 μc/kg of plutonium (T17P5). ×600.

Fig. 24. Appearance of pericemental fibrosis and basophilic staining acellular cementum lacking cementoid layer 777 days after injection of 3·0 μc/kg of plutonium (T7P5). ×60.

Fig. 25. Normal appearance of cellular cementum, cementoid tissue and cementoblasts in a 17 month-old control (M35A). ×330.

Fig. 26. Abnormal cellular cementum with basophilic staining post injection cementum with empty lacunae and pericemental fibrosis 777 days after injection 3·0 μc/kg of plutonium (T7P5). ×330.
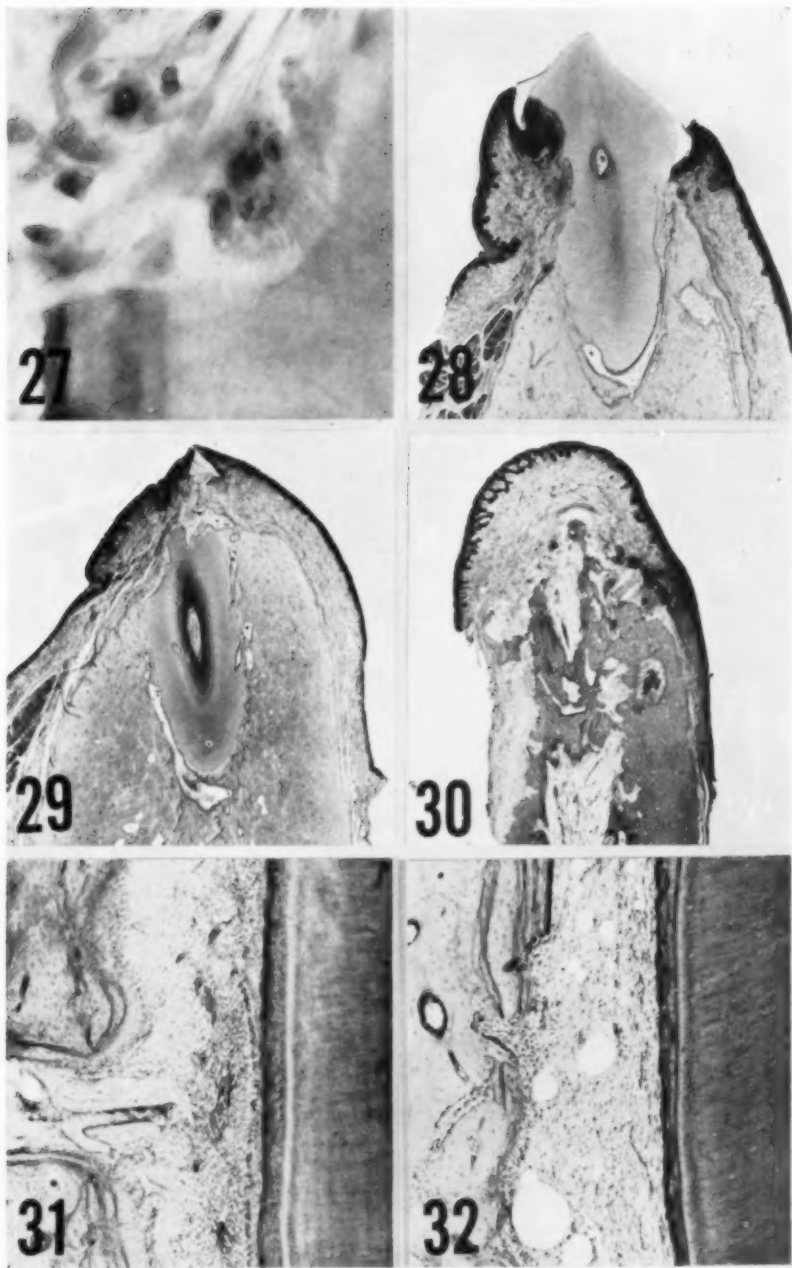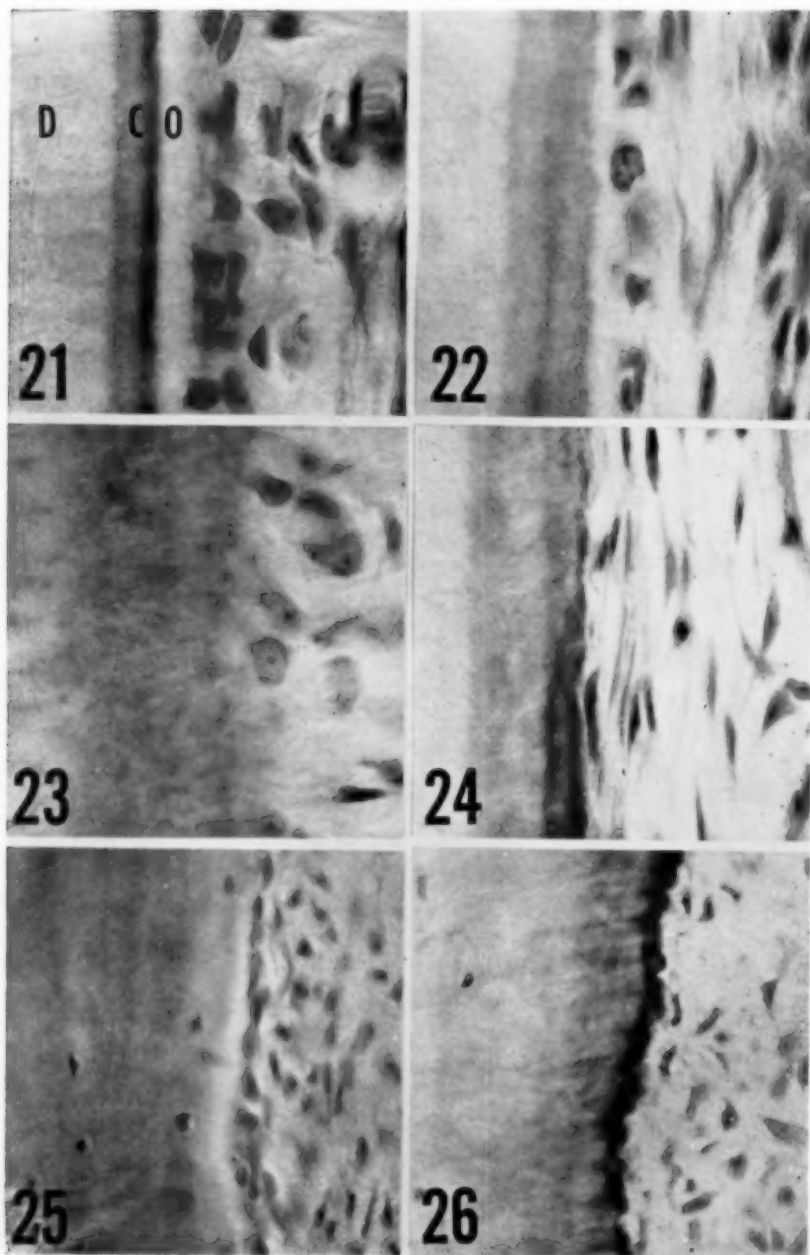
Plate 6

PLATE 5

PLATE 6

FIG. 27. An osteoclast in Howship lacuna in dentine (T7P5). ×600.

FIG. 28. Labiolingual section of premolar showing massive resorption in neck of tooth with replacement by gingiva. ×5·4.

FIG. 29. Labiolingual section of premolar showing loss of crown but with the root remaining ankylosed to mandible. ×5·4.

FIG. 30. Labiolingual section of premolar showing the loss of tooth, massive resorption of mandible with fibrosis and overgrowth of new gingival and bone tissue. ×5·4.

FIG. 31. Normal appearance of alveolar bone, periodontal ligament with blood vessels, cementoblasts, cementoid tissue, cellular cementum and dentine in control dog. ×132.

FIG. 32. Appearance of identical region as Fig. 31 in dog containing plutonium (T7P5). Note basophilic borders on alveolar bone and cellular cementum, pericemental fibrosis, dilated vessels in periodontal ligament and periodontal fibres orientated parallel to mineralized structure. ×132.

PLATE 7

FIG. 33. Labiolingual section of molar showing secondary dentine, lamellar bone, gingival replacement of dentine and ankylosis. ×3.

FIG. 34. Enlargement of area of dentine containing lamellar bone from Fig. 33. Note the concentric arrangement of one of the deposits resembling a Haversian system. ×156.

FIG. 35. Labiolingual section of molar showing the appearance of secondary dentine in pulp cavity as the result of the massive resorption in the region of the neck. ×3.

FIG. 36. Normal appearance of labiolingual section of molar and mandible in control dog. ×3.

FIG. 37. An osteogenic sarcoma located in a portion of the mandible in dog injected with radium (M7R5). ×3.

PLATE 7

SHORT COMMUNICATIONS

DENTAL CALCULUS IN THE GERM-FREE RAT

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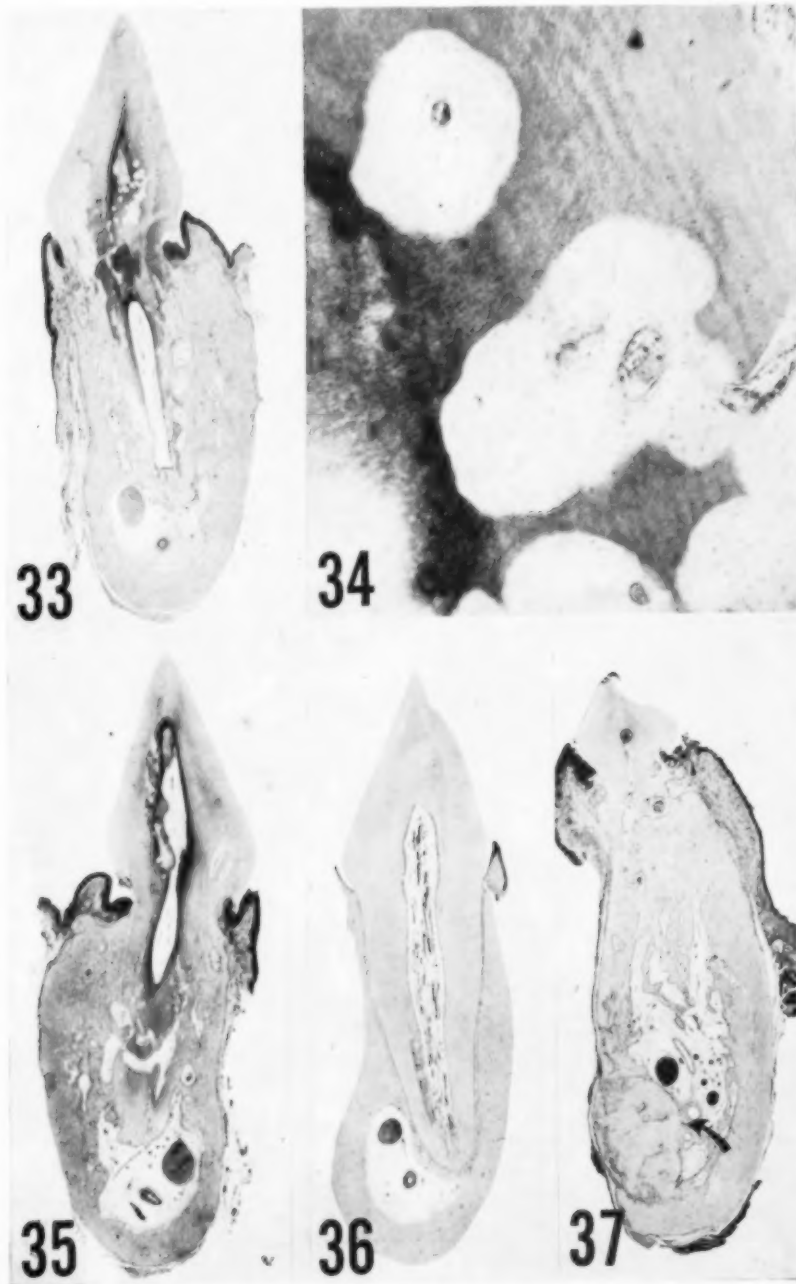
The calcareous deposits found on the molar teeth of white rats have been shown to contain apatite (Fitzgerald, Jordan, Scott and McCann, 1960), the predominant mineral species in human dental calculus (Leung and Jensen, 1958). Because there has been considerable speculation on the role of micro-organisms in the formation of dental calculus (Mühlemann and Schneider, 1959; Gonzales and Sognnaes, 1960), it seemed desirable to extend some observations (Fitzgerald, 1959; Fitzgerald, Jordan and Stanley, 1960) suggesting that dental calculus deposition could occur in the germ-free rat. Recently Baer and Newton (1959) have reported the occurrence on the maxillary molars of germ-free mice of hard, alizarin-staining deposits which they called calculus.

In post-mortem examinations of formalin-fixed jaws of germ-free rats which had been maintained on a variety of steam-sterilized diets we have routinely observed the presence of an adherent pellicle (Vallotton, 1945) on the first maxillary molars of most of the animals. The texture of the pellicles ranged from leathery to hard and brittle, and they stained readily with 0·02% alizarin red S. (Fig. 1).

The deposits were confined mainly to the mesial surface of the first maxillary molar but occasionally could be found on the buccal surface of the second cusp of this tooth in addition. In size and texture the pellicles resembled those seen in conventional (non-germ-free) rats on cariogenic diets (Fitzgerald et al., 1960). In the limited germ-free material examined thus far, the impression was gained that the texture of the pellicle could vary considerably on any one diet, although none of the germ-free animals had received a diet comparable in effect to that (Fitzgerald et al., 1960) which resulted in heavy calculus deposits in conventional rats. When the leathery pellicles were examined in the polarizing microscope, birefringent areas were rarely seen, indicating the presence of little or no crystalline material. However, a sufficient amount of the brittle pellicular material was collected from the first maxillary molars of six germ-free rats which had been maintained on a folic acid-deficient diet to enable X-ray diffraction studies to be performed. The patterns obtained, using copper radiation, coincided with those of authentic apatite. (We are indebted to Vernon R. Mosely for the X-ray diffraction analysis.)

The animals sampled consisted of three male and three female rats of the Lobund germ-free strain. They were received at weaning age from the Lobund Institute and placed on the diet shown in Table 1 for an average period of 90 days. At the

TABLE 1. COMPOSITION (BEFORE STERILIZATION) OF FOLIC
ACID-DEFICIENT DIET PER 100 g

Corn starch	69·0 g	Niacinamide	10·0 mg
Casein (vitamin free)	22·0 g	Biotin	5·0 µg
Salt mixture(W)	4·0 g	Choline Cl	200 mg
Crisco	5·0 g	Vitamin B_{12}	10 µg
Thiamine HCl	1·0 mg	Vitamin A	12,000 I.U.
Pyridoxine HCl	1·0 mg	Vitamin D	2500 I.U.
Riboflavin	2·0 mg	Vitamin E acetate	5·0 mg
Ca pantothenate	2·0 mg	Menadione	0·2 mg

termination of the experiment the heads were fixed in 10 % buffered formalin, following which the mandibles were removed and the maxillary molars were cleansed of soft debris by means of a soft brush in running water. The pellicles were carefully removed with a fine dental explorer and pooled for the diffraction analysis.

Periodic bacteriological checks on the food and excreta of the animals revealed no viable micro-organisms, and Gram-stained smears of decalcified deposits confirmed their absence. Thus it seems apparent that dental calculus deposition can occur in the germ-free rat in the absence of a microbial flora. This observation does not rule out microbial participation in dental calculus formation in conventional animals where the oral microflora is ever present. Indeed, it seems logical to expect that the presence of micro-organisms would influence the nature or extent of calcification. In this connection WASSERMAN, MANDEL and LEVY (1958) have demonstrated that colonies of *Actinomyces israelii* of human oral origin could calcify in an *in vitro* test system, and that calcification occurred even when the organisms had been killed by prior treatment with formalin. The findings in the present study with germ-free animals, however, indicate that dental calculus deposition does not necessarily depend on the presence of micro-organisms either living or dead.

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FIG. 1. First mandibular molar of germ-free rat showing alizarin stained deposits on the mesial and buccal surfaces of the first two cusps. Part of the mesial deposit has been chipped off with an explorer.

PLATE 1

THE EFFECT OF PAPAIN ON THE STAINING PROPERTIES OF BONE AND TEETH

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THE present writer has shown that, after extraction of hard tissues with pyridine, Sudan black will stain specifically the sites where calcification is being initiated (IRVING, 1959). The nature of the staining material is not known, but circumstantial evidence previously presented made it seem possible that it was associated with mucopolysaccharides.

Papain is reported to cause a release of chondroitin sulphate from cartilage (BRYANT, LEDER and STETTEN, 1958), due to its action on the protein to which the chondroitin sulphate is attached. Concurrently a very characteristic change occurs in the epiphyseal cartilage of growing bone leading to the arrest of calcification (HULTH and WESTERBORN, 1959).

This communication reports some preliminary experiments in which papain was given to young growing rats to see if it affected in any way the Sudan black staining of bone and teeth. Seven litter-mate albino rats of the Wistar Institute strain, weighing about 100 g each, were used. One was killed at the commencement of the experiment, and the rest were injected twice daily intraperitoneally with a 1 % solution of crude papain (Nutritional Biochemicals Corp.) at a dose level of 100 mg dry enzyme preparation per kilogram body weight. The rats were maintained on a normal diet and appeared perfectly healthy throughout the experiment. They were killed at daily intervals; the upper halves of the tibias and the upper incisor teeth with the surrounding bone were removed, sectioned and stained with haematoxylin and eosin, with toluidine blue, or with Sudan black, the latter according to IRVING's method (1959).

The resulting effects, while becoming more severe with the passage of time, were qualitatively the same in all animals.

Endochondral bone formation. The animal killed before the experiment began had normal tissues. In particular the matrix around the hypertrophic cartilage cells stained strongly with Sudan black, and was much more metachromatic than the rest of the cartilage, which did show a high degree of metachromasia.

Even after one day's injections, the epiphyseal cartilage had narrowed, all layers appearing to be reduced in width. The earlier cells were beginning to bunch into nests. The epiphyseal cartilage was still moderately metachromatic, but the marked metachromasia around the hypertrophic cells had disappeared. Sudan black still stained the matrix between the hypertrophic cells, indicating that calcification was still in progress.

With the passage of time, the orderly arrangement of epiphyseal cartilage cells was lost, the cells being clustered into small groups. In addition, they all looked alike, having changed to small rounded cells. Relatively more matrix than normal was present. The cartilage was slightly metachromatic at the second day, but thereafter was not metachromatic at all. The cells stained a dull grey colour and the matrix was unstained with toluidine blue. Sudan black staining of the calcifying cartilage matrix became gradually less with time, but did not completely disappear till the sixth day.

Intramembranous bone formation. Bone formation on the shaft of the tibia, and on the bones of the skull (the horizontal process of the palatine bone was especially studied) appeared to be completely unaffected by papain. Calcification lines staining with Sudan black were present at the edge of the pre-osseous matrix in all animals, and these were more metachromatic than the rest of the bone, as has also been reported by VINCENT (1955).

Incisor teeth. Enamel formation and calcification were unaffected by the papain injections. Dentine formation was slightly affected to the extent that rather more stratification was seen in the dentine after the injections began, but the predentine width was normal and toluidine blue and Sudan black staining the same as in control sections.

It appears from these results that if mucopolysaccharides are involved in the calcification of teeth and intramembranous bone they are unaffected by papain. It would also seem that chondroitin sulphate is rapidly mobilized from the epiphyseal cartilage, as evidenced by the loss of metachromasia. This was not associated with a simultaneous cessation of calcification, and in fact the stoppage of calcification seemed to be more due to the upset in the organization of the cartilage, the formation of hypertrophic cells being suppressed. Under the circumstances of this experiment, the chondroitin sulphate which is mobilized by papain was not associated with the calcification process, nor was it stained by Sudan black.

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THE ULTRASTRUCTURE OF AMELOBLASTS FROM THE GROWING END OF RAT INCISORS*

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Abstract—Ameloblasts from the growing end of rat incisors were examined with the electron microscope. They exhibit marked polarity with the nucleus at the basal end of the cell. Mitochondria are proximal to the nucleus. Just distal to the nucleus is a well defined Golgi apparatus. Rough-surfaced endoplasmic reticulum and granules fill the remainder of the cytoplasm. Some 150 Å particles are independently present in the cytoplasm.

The cells are close to each other with a minimum of extracellular space. Terminal bars are present at the junction of the ameloblasts with Tomes' process. Each cell contributes a cell membrane and opaque cytoplasm to the terminal bar.

At least two components are seen in enamel matrix: small rodlets 60 Å wide and up to 1000 Å long, and a more extensive amorphous material.

Ameloblasts from a more incisal location are separated by a large extracellular space into which microvilli project. In the most incisal region examined, small granules measuring 70 Å are found in the ameloblasts and the cells are connected by desmosomes.

INTRODUCTION

THE incisor teeth of rats are elongated curved structures, orientated in an anterior–posterior direction. They can be removed with reasonable ease and possess a particular attractiveness to the investigator in that they undergo continuous growth and eruption (SCHOUR, 1932). The enamel of these teeth is pigmented, and is present only on the convex surfaces (Fig. 1). Although mature enamel is hard, along with other tooth structures, it is subject to continual loss in the oral cavity by attrition. The lost tooth structures are replaced by the constant production of new tissue. This begins at the growing end of the tooth and includes the formation of enamel matrix by tall columnar ameloblasts of the enamel organ. The enamel matrix is soft and can be cut without the use of decalcifying agents. As the newly formed matrix moves incisally, it undergoes certain changes termed maturation which include an influx of calcium salts and a decrease in water (DEAKINS, 1942). The final product is hard mature enamel. The ameloblasts retain their close relationship to enamel during maturation and remain in contact with it as far as the oral epithelium.

The manner whereby enamel is formed has been a subject of considerable controversy through the years (CHASE, 1932; MARSLAND, 1951; ORBAN, SICHER and WEINMANN, 1943; SAUNDERS, NUCKOLLS and FRISBIE, 1942). While it is known that the process consists of two phases, matrix formation and subsequent maturation (MARSLAND, 1952; ORBAN, 1953), much remains to be learned about the details of

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each. New methods are supplying much information in these areas. The contributions of electron microscopy to tooth biology up to 1953 have been reviewed by SCOTT (1953). The bulk of this early work dealt with the structure of enamel and dentine. In more recent years accounts of cellular ultrastructure during tooth formation have appeared (LENZ, 1957; NYLEN and SCOTT, 1958a, b; QUIGLEY, 1959a, b; WATSON and AVERY, 1954). The descriptions of ameloblasts have been rather sketchy in these papers either because the main emphasis was directed to some other aspect of tooth structure, or the report appeared in abstract form. In any event, there is agreement that in the differentiated ameloblast the mitochondria appear proximal to the nucleus and endoplasmic reticulum is distally located. QUIGLEY (1959a) was unable to find cell membranes and terminal bars in the distal region of hamster ameloblasts and considered the possibility that these cells formed a syncytium. Although the Golgi apparatus of ameloblasts has been observed with the light microscope (BEAMS and KING, 1933), it has not been mentioned in the above papers.

The present paper consists of a report on the structure of ameloblasts from the growing end of rat incisors. Fig. 1 shows the region from which the cells were taken. A number of features not previously observed with the electron microscope will be presented. Information will also be presented on the structure of enamel matrix, and some changes which occur in ameloblasts as they move incisally.

MATERIALS AND METHODS

Adult rats were sacrificed with an overdose of ether. The incisors with adherent enamel organs were removed and placed into chilled 1% osmium tetroxide, buffered to pH 7·2 with veronal acetate (PALADE, 1952). The time required for the removal of the first upper incisor was invariably less than 1 min, while the whole procedure for the four teeth took from 10 to 15 min. Following 2 hr in fixative, they were placed into water at which time each tooth was further trimmed. The tissues were then dehydrated through a graded series of alcohols and infiltrated with methacrylate (NEWMAN, BORYSHO and SWERDLOW, 1949) in gelatin capsules, and oriented in such a way that the knife would cut ameloblasts in a transverse or slightly oblique plane. Polymerization was accomplished at 56°C with Luperco as a catalyst. Thin sections were cut with glass knives on a Porter-Blum microtome. They were mounted on formvar coated copper grids and examined in an RCA EMU 2e or an EMU 3a electron microscope.

Some sections were treated with saturated uranyl acetate for 1 hr prior to examination to enhance contrast (WATSON, 1958).

RESULTS

The terminology used to describe ameloblasts in this paper takes into consideration the repolarization which they have undergone (BEAMS and KING, 1933). Thus the part of the cell adjacent to the stratum intermedium is referred to as proximal or basal, and the part facing enamel is referred to as distal.

Differentiated ameloblasts are lined up in an orderly fashion with their nuclei in the basal ends of the cells (Fig. 1). A small amount of cytoplasm is proximal to the

nucleus, while the largest amount is distally located. When viewed with the electron microscope, ameloblasts present an array of cell organelles similar to those which have been identified in other cell types.
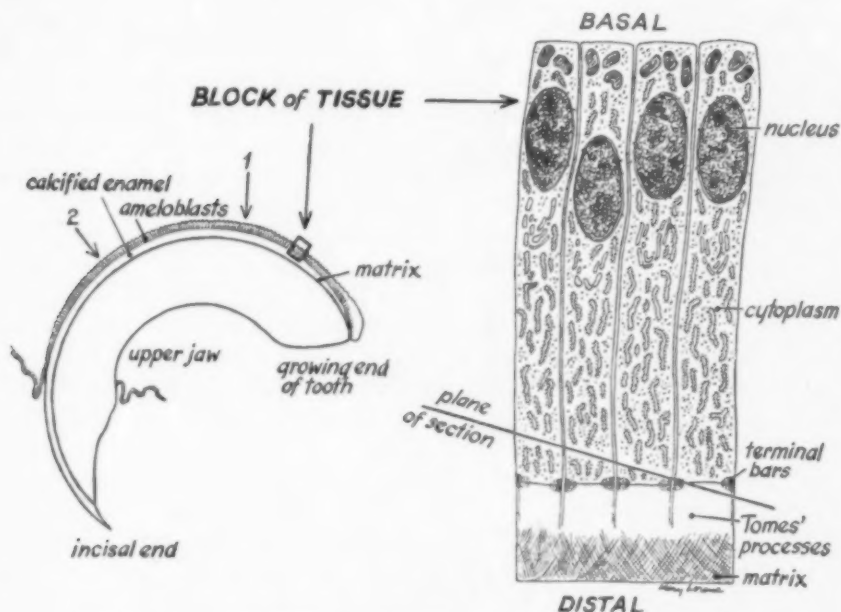
Fig. 1. Diagram of upper incisor of rat. Tissue studied was taken from region marked by rectangle. Enlargement of rectangle shows ameloblasts, Tomes' process, and matrix, but not stratum intermedium or ridges. Plane of section is indicated in the diagram. The appearance of a section cut in such a plane is shown in Fig. 2. Other sections were cut at different levels in planes parallel, or nearly parallel, to the one illustrated.

Cell membranes. In transverse sections, the ameloblasts appear closely applied to each other with only a minimal amount of space between them (Figs. 2, 5). Cell membranes can be clearly identified at all levels of the cell. They are uncomplicated, and define the polygonal shape of the ameloblast. Occasionally the membranes of neighbouring cells separate slightly to outline a section of intercellular space (Fig. 6).

At the distal end of the ameloblast, a specialization of the cell membrane occurs in the form of terminal bars (Figs. 2, 3, 4). In oblique sections, these are seen to consist of two neighbouring cell membranes and adjacent opaque cytoplasm. Each membrane measures about 50 Å in width. The distance between them is 80 Å. Next to each cell membrane there is a narrow border of opaque cytoplasm, ranging from 200 to 400 Å in width. A broader band of opaque material, less regularly present, also exists. This may be as wide as 0.4μ.

Cytoplasm. Rough-surfaced endoplasmic reticulum can be seen in all levels of the ameloblast. Most of it, however, is located in the distal end of the cell (Figs. 2,

5, 6), where it is the predominant feature. It extends as far as the terminal bars (Fig. 2). In transverse sections, the endoplasmic reticulum appears as elongated profiles of two parallel membranes which meet at both extremities. Between the two membranes is a clear space. Small particles measuring from 150 to 250 Å stud the outer surface of the membranes. The inner surface is smooth. Similar particles are also independently scattered throughout the cytoplasm. Less regular forms of the endoplasmic reticulum with attached particles are also seen.

A Golgi apparatus exists just distal to the nucleus. Sections through this level show it to be present in every cell. It is centrally located, presenting a circular or oval outline. The predominant features of this structure (Fig. 7) are smooth-surfaced parallel membranes about 50 Å wide and small vesicles. The parallel membranes meet at both extremities. Gradations ranging from vesicles to flattened profiles can be seen in most sections. In addition, Golgi granules are present with sufficient frequency to suggest that they are a part of each complex. They vary in size; one shown in Fig. 7 consists of a dense granule about $0 \cdot 1$ μ dia., surrounded by a smooth surfaced irregular membrane.

Mitochondria are present in the basal end of the cell, mostly proximal to the nucleus. They are recognized by the presence of an outer double membrane, and a system of internal membranes. The mitochondria of differentiated ameloblasts are closely packed (Fig. 8), leaving little space for other organelles. They vary in form, and may be as much as 2 μ long.

Lipid bodies are occasionally seen in ameloblasts, distal to the nucleus. They may be present in sections through the Golgi region or they may be more distally located. They usually measure about 1 μ (Fig. 9).

In addition to the above structures, ameloblasts contain a variety of granules, four of which are shown in Fig. 10. All appear to be surrounded by a fine membrane. For purposes of description, they will be designated A, B, C and D. Granule A contains a light amorphous material which fills most of the granule. It also contains a group of small circular osmiophilic bodies, 400–500 Å in diameter. The contents of granule B are darker than those of granule A. No internal structure can be discerned here. Granule C consists of a dispersion of fine granular material more opaque than A or B. Granule D is the most dense of the four. It consists of opaque amorphous material. The size of the granules which occur in ameloblasts varies considerably. Although in Fig. 10 they all measure from 1 to $1 \cdot 5$ μ, other sections reveal granules of different sizes, some smaller, some larger (Fig. 2). In most cases they can be related to the four types described above. All types are found with considerable frequency in the Golgi region.

Nuclei. The nuclei consist of granular material (Fig. 11). They are surrounded by two osmiophilic membranes each about 90 Å wide (Fig. 12). Between the membranes is a lighter band which varies in thickness. In many places small particles are attached to the outer membrane. These range from 150 to 200 Å and are probably similar to particles of the same size in other regions of the cell. The apparent absence of a nucleus in the cell in the centre of Fig. 11 is due to the fact that not all nuclei are at exactly the same level.

Enamel matrix. Enamel matrix is regularly obtained along with enamel organ in sections from the growing end of the rat's incisor. Unfortunately, the angle at which the sections were cut in the present study makes it difficult to analyse the relationship of matrix to Tomes' process and the ameloblasts. This is best accomplished by means of longitudinal sections. However, the present material does permit a consideration of matrix structure.

The relatively low magnification in Fig. 2 offers little opportunity to study constituents of matrix. Higher magnifications show at least two components (Figs. 13, 14): a dense rod-shaped component (rodlet), and a more extensive amorphous material of varying opacity. The rodlets are orientated in two general patterns. In one pattern, they have a crude "herringbone" relationship to each other. In the other, they are arranged with their long axes in the same direction. They measure about 60 Å wide and may be as long as 1000 Å. Many have a light band about 30 Å wide along their sides. The more extensive amorphous material is light and finely dispersed where it is free of rodlets, otherwise where it forms a background for the rodlet it is more opaque. This material may condense into forms which have the same shape as rodlets. Moreover, a condensation of this material may be seen bordering some light bands. It is most conspicuous when two rodlets with their light bands are parallel. In these cases it appears as a dark band as little as 20 Å wide between two neighbouring light bands.

Alterations in ameloblasts. The descriptions given above apply to tissue taken from the region of the tooth as indicated by the rectangle in Fig. 1. Marked differences exist in ameloblasts somewhat more incisally located (Fig. 1, arrow 1). Most conspicuous is the relatively large amount of intercellular space which now exists between the cells (Fig. 15). Microvilli extend into this space. The cytoplasm contains some double membranes and an assortment of vesicles and granules of various sizes.

More incisally still (Fig. 1, arrow 2) ameloblasts contain aggregates of small dense granules about 70 Å in diameter. Typical desmosomes connect cells in this region (Fig. 16).

DISCUSSION

Cell membranes were described in young columnar ameloblasts (internal enamel epithelium) before the onset of enamel matrix formation (LENZ, 1957) and in the present report in active differentiated cells. The failure to find them in hamster teeth (QUIGLEY, 1959a) could be due to the presence of a more highly active state in which their identification is difficult. Certainly a marked change in appearance occurs as ameloblasts move incisally. Ameloblasts from the growing end of the rat's tooth, as illustrated in this report, are conspicuous for their closeness (Fig. 5). It would seem that very little could pass between them. This is in sharp contrast to the arrangement of ameloblasts obtained from a more incisal area (Fig. 15). Here there is ample extracellular space which would permit exchange between cell and space. The presence of microvilli suggests that this is the case.

The presence of opaque 70 Å granules in more incisally located ameloblasts is expected. Aggregations of these small granules appear as yellow pigment with the

light microscope. This yellow material has been shown to contain iron by chemical and histochemical analysis (IRVING, 1952; REITH, 1959). It is significant that examination of ferritin and haemosiderin with the electron microscope shows they contain opaque granules which also measure 70 Å (RICHTER, 1958).

The terminal bars as observed here with the electron microscope are the same structures which have been observed with the light microscope (ORBAN, SICHER and WEINMANN, 1943). They are seen to consist of parallel neighbouring cell membranes with a border of opaque cytoplasm. No thickening of the cell membrane was observed as described by FAWCETT (1958) and YAMADA (1955). It is unlikely that treatment with uranyl acetate causes the membranes to appear thinner since no noticeable difference, apart from contrast, was observed in untreated sections. Moreover, whereas molybdate is reported to cause a thinning of membranes (WATSON, 1958), no such thinning was ascribed to uranyl acetate. It was concluded by RHODIN and DALHAMN (1956) that the opaque cytoplasm was involved in adherence of the cells. The findings of the present investigation are consistent with such an interpretation.

The work of earlier investigators (NYLEN and SCOTT, 1958b; QUIGLEY, 1959a, b; WATSON and AVERY, 1954) offers little information on the structure of the endoplasmic reticulum and associated particles in the differentiated ameloblast. Considering the report by NYLEN and SCOTT (1958b) that this organelle is longitudinally arranged, it is very likely that the present illustrations represent cross sections of this structure. The present results do not suggest two patterns of orientation as described by QUIGLEY (1959b). The size of both free and attached particles in the present paper compares with the size of similar particles described by SJÖSTRAND and HANZON (1954a) and PALADE (1955), and subsequently related to RNA (PALADE and SIEKEVILZ, 1956). There can be little doubt that the cytoplasm of ameloblasts is rich in RNA in view of the histochemical findings (JOHNSON and BEVELANDER, 1954; SYMONS, 1956; WISLOCKI and SOGNNAES, 1950). Considering the readily demonstrable basophilia (CHASE, 1932; MARSLAND, 1951, 1952; ORBAN, SICHER and WEINMANN, 1943; SAUNDERS, NUCKOLLS and FRISBIE, 1942), the histochemical demonstration of RNA and the compatable electron microscopic appearances, it would be appropriate to refer to this organelle as ergastoplasm.

The Golgi apparatus described in this paper is located in the same region as was previously observed (BEAMS and KING, 1933) with the light microscope. A Golgi apparatus occupies basically the same position in the odontoblast (NYLEN and SCOTT, 1958a). It is significant that whereas the production of enamel matrix is interpreted as a transformation of the ameloblast, and is compared to the process of keratinization (QUIGLEY, 1959a; WATSON and AVERY, 1954), there is no conspicuous Golgi apparatus in cells undergoing keratinization (ROGERS, 1959; SELBY, 1955). On the other hand, a Golgi apparatus similar to the one described here is found in other cells (DALTON and FELIX, 1956; RHODIN, 1954; SJÖSTRAND and HANZON, 1954b).

Mitochrondrial structure has been described by a number of workers (PALADE, 1953; RHODIN, 1954; SJÖSTRAND, 1953; SJÖSTRAND and RHODIN, 1953). Their position in ameloblasts agrees with the findings of earlier investigations with the

electron microscope (NYLEN and SCOTT, 1958b; QUIGLEY, 1959b; WATSON and AVERY, 1954). The small lipid bodies are probably related to the sudanophilic material observed in the distal end of the cell with the light microscope (WISLOCKI and SOGNNAES, 1950). The wide variety of large granules is hard to interpret. An interpretation of their morphological and functional relationship will be more valid following examination of ameloblasts from all regions of the enamel organ.

The picture of enamel matrix as presented in this paper should be considered as a preliminary report. Unfortunately, electron diffraction patterns were not available at the time the paper was submitted. In the absence of such data, any opinion on the nature of the small rodlets is premature. However, it is interesting that the size of these structures is comparable with the size of young apatite crystals (NEUMAN and NEUMAN, 1958). Fibrous material has not been seen in these preparations of enamel matrix. It could very well be that the amorphous material represents young material which becomes fibrous as it matures. The term rodlet is used rather than rod lest there be confusion with enamel rod which has a definite meaning in dental histology.

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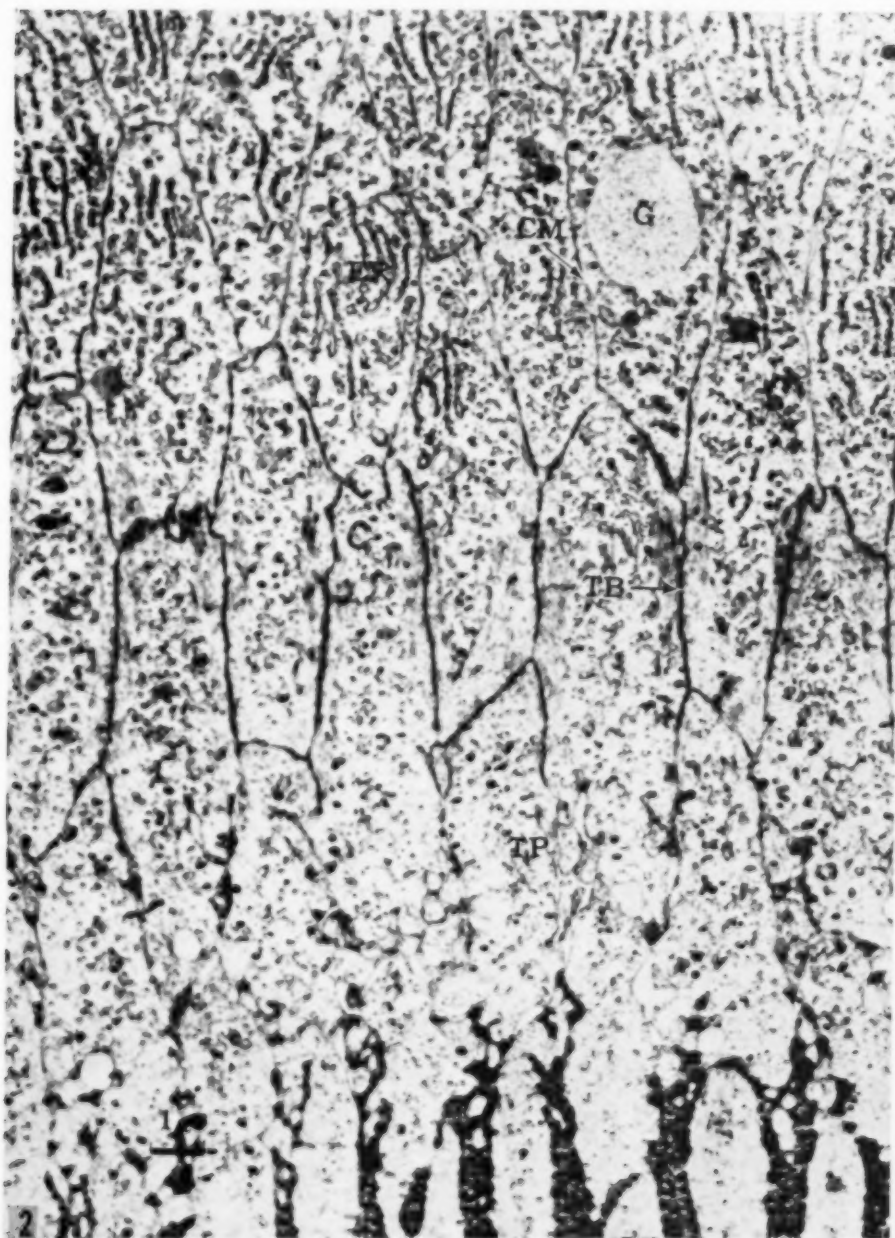
FIG. 2. Oblique section through distal end of ameloblasts, terminal bars, Tomes' process, and enamel matrix. CM=cell membrane; ER=rough-surfaced endoplasmic reticulum; G=granule; TB=terminal bar; TP=Tomes' process. Note how endoplasmic reticulum ends at level of terminal bars. Enamel matrix is at the bottom. ×7200.

PLATE I
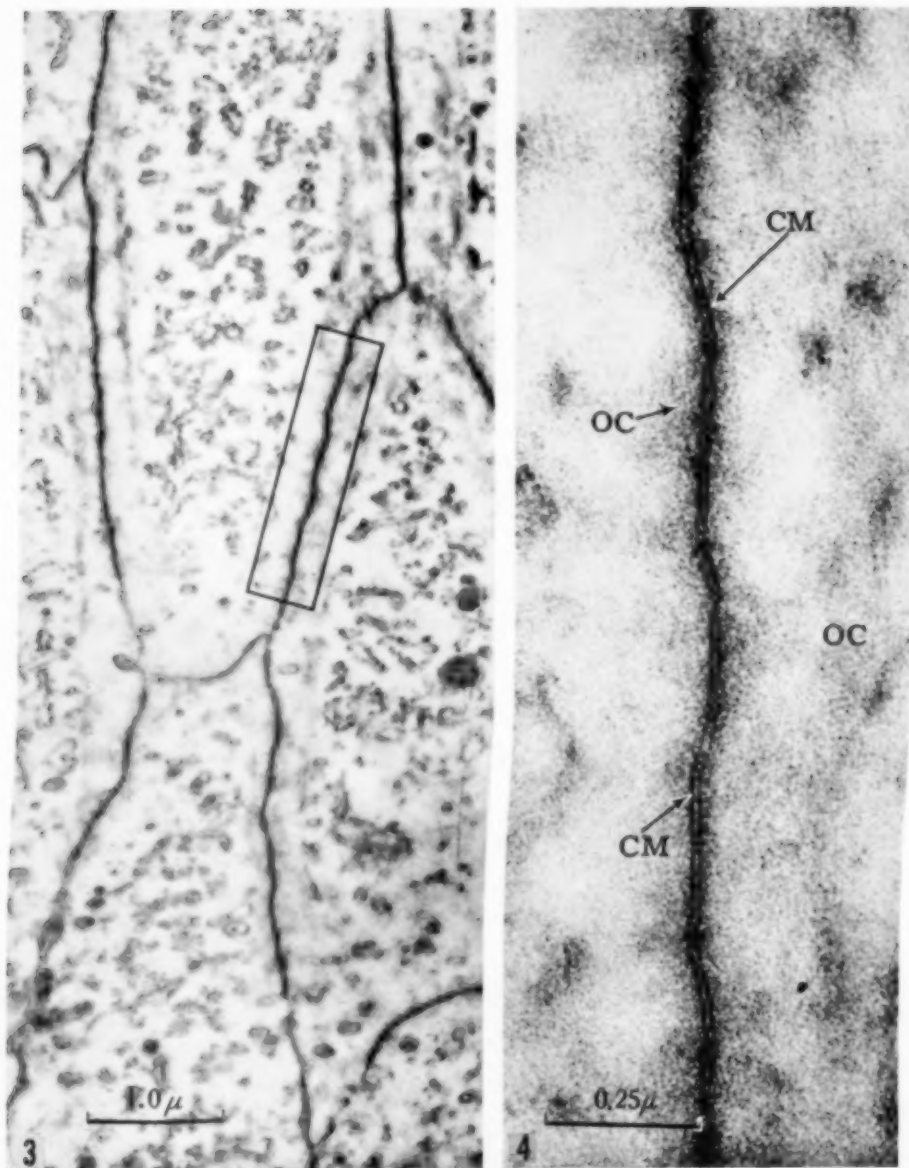
Fig. 3. Oblique section through terminal bars. This section was treated for 1 hr with saturated uranyl acetate prior to examination. Rectangle shows area which was enlarged in Fig. 4. ×19,000.

Fig. 4. High magnification of terminal bar. Arrow points to the narrow band of opaque cytoplasm (OC) just adjacent to the cell membrane (CM). This section was treated for 1 hr with saturated uranyl acetate prior to examination. ×90,000.

PLATE 2
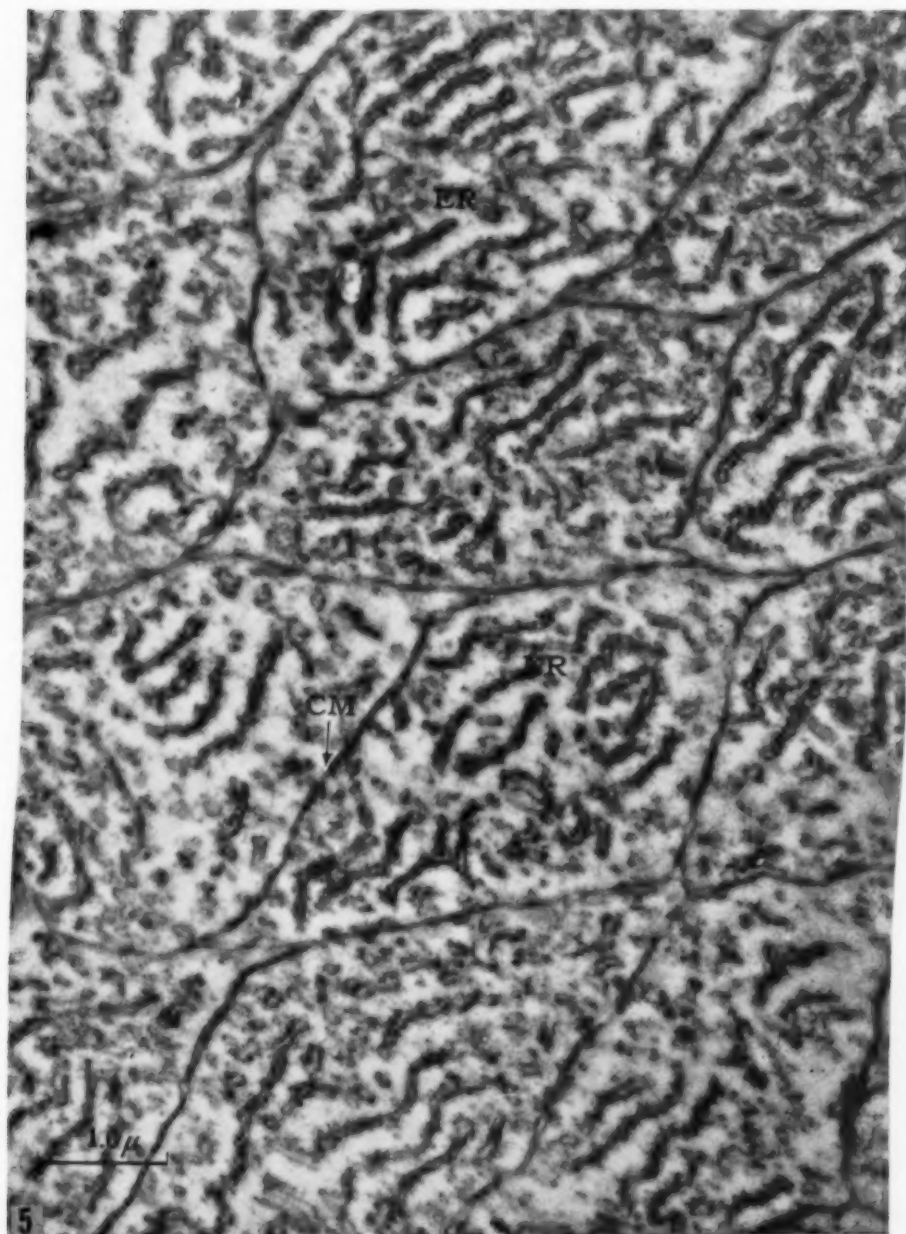
Fig. 5. Section through distal region of ameloblasts. Neighbouring cell membranes (CM) are very close. Rough-surfaced endoplasmic reticulum (ER) is the predominant organelle at this level. ×19,000.

PLATE 3
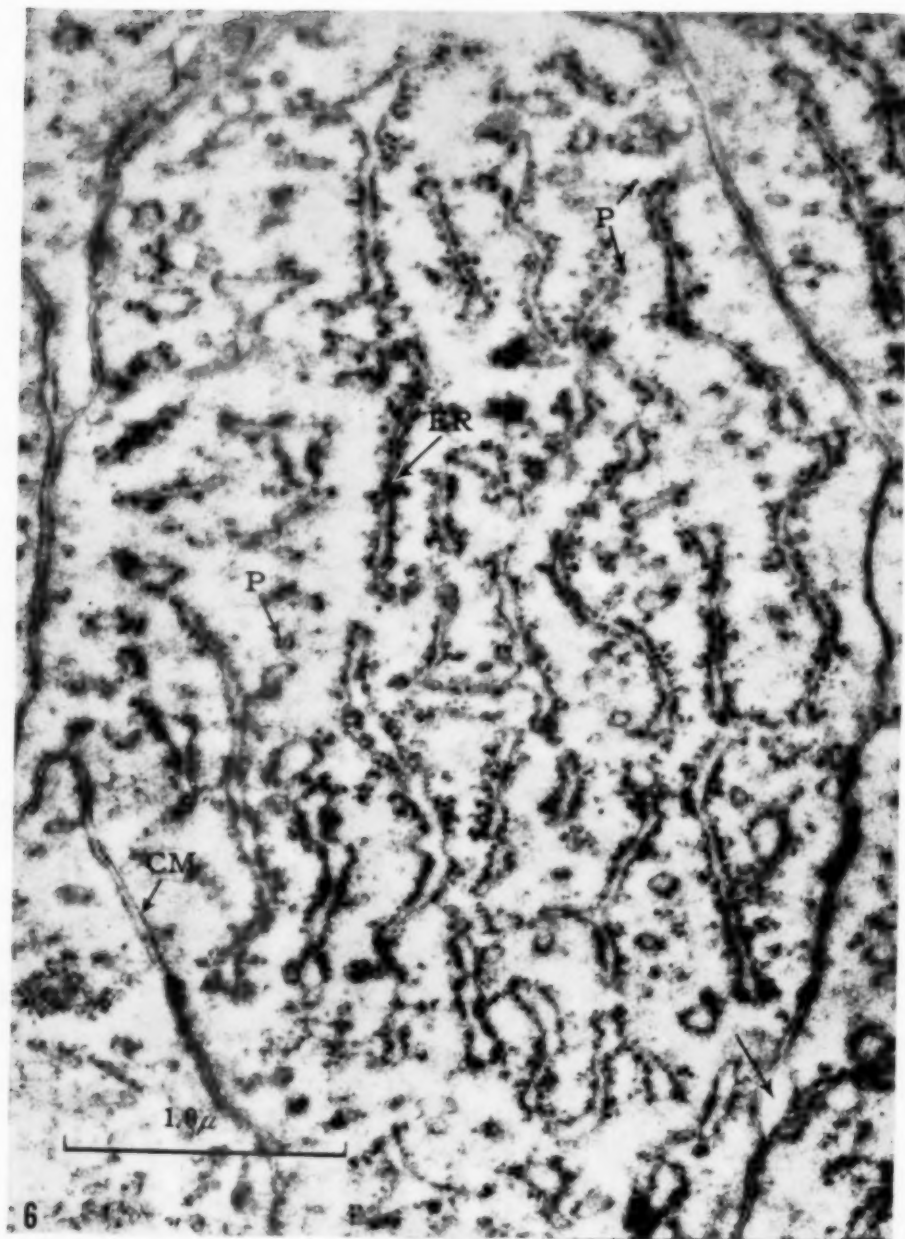
FIG. 6. Distal end of ameloblast showing rough-surfaced endoplasmic reticulum (ER) and independent small particles (P). The unmarked arrow shows where cell membranes (CM) separate to outline a small section of extracellular space.   ×40,000.

PLATE 4
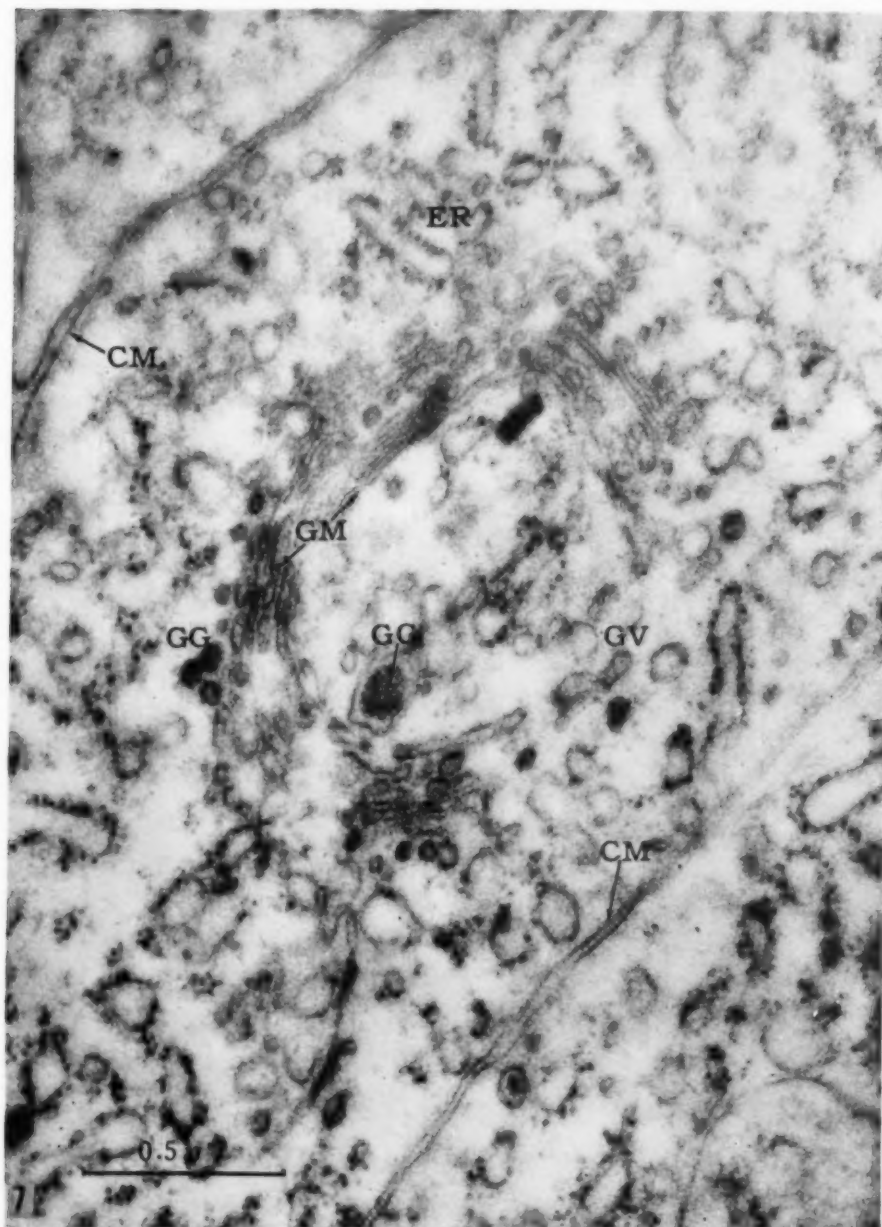
FIG. 7. Golgi apparatus in centre of ameloblast. CM=cell membrane; ER=rough-surfaced endoplasmic reticulum; GG=Golgi granules; GM=Golgi membranes; GV=small Golgi vesicles. ×58,000.

PLATE 5
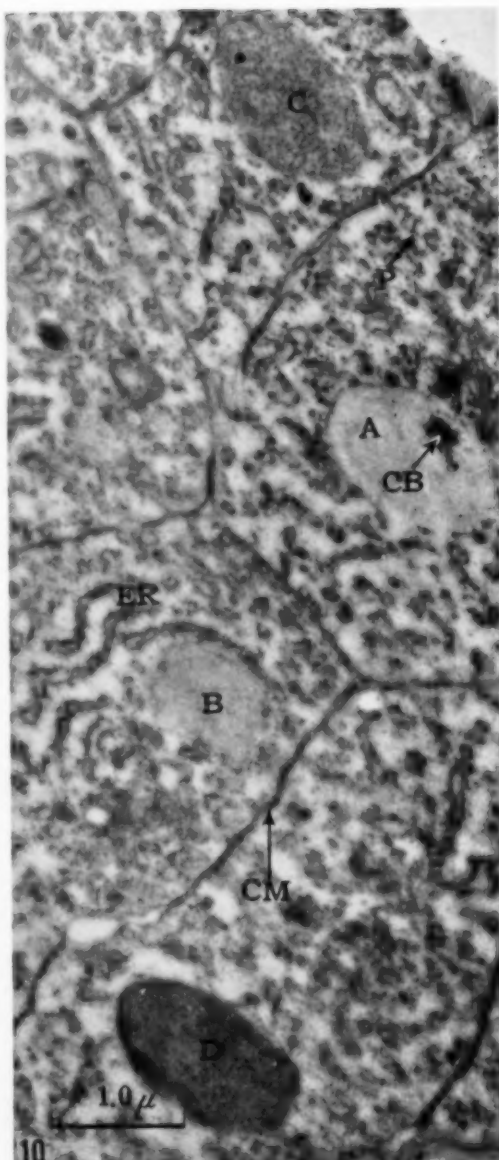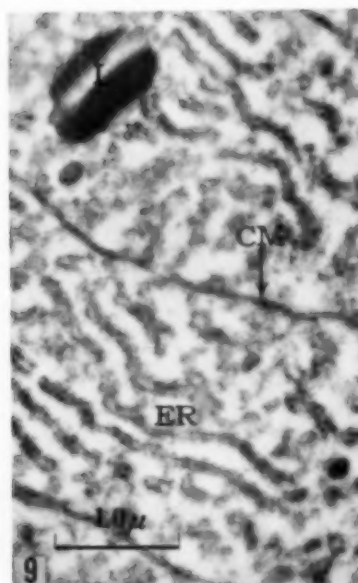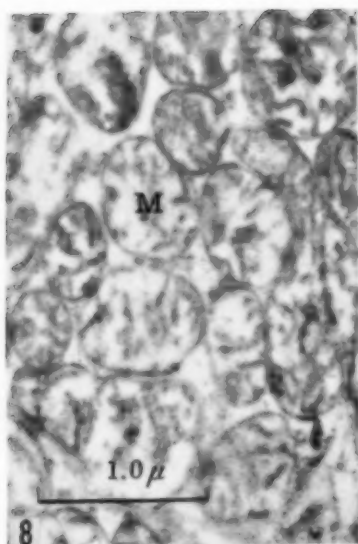
Fig. 8. Mitochondria (M) from level proximal to nucleus. ×24,000.

Fig. 9. Distal end of ameloblast showing lipid body (L). ×19,000.

Fig. 10. Section through distal half of ameloblasts showing large granules A, B, C and D, and circular bodies (CB) in granule A. ×19,000.

PLATE 6
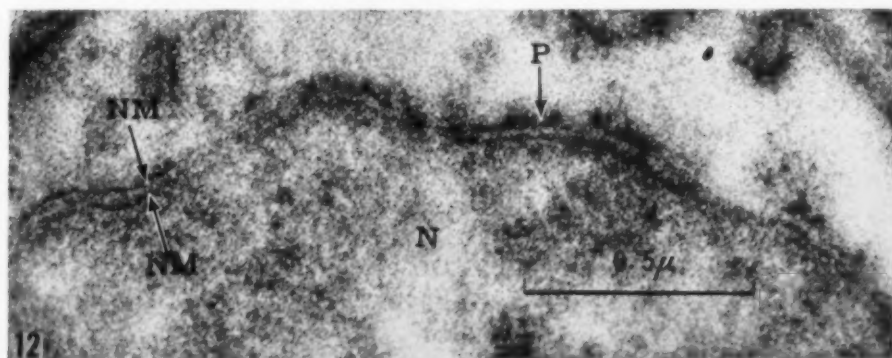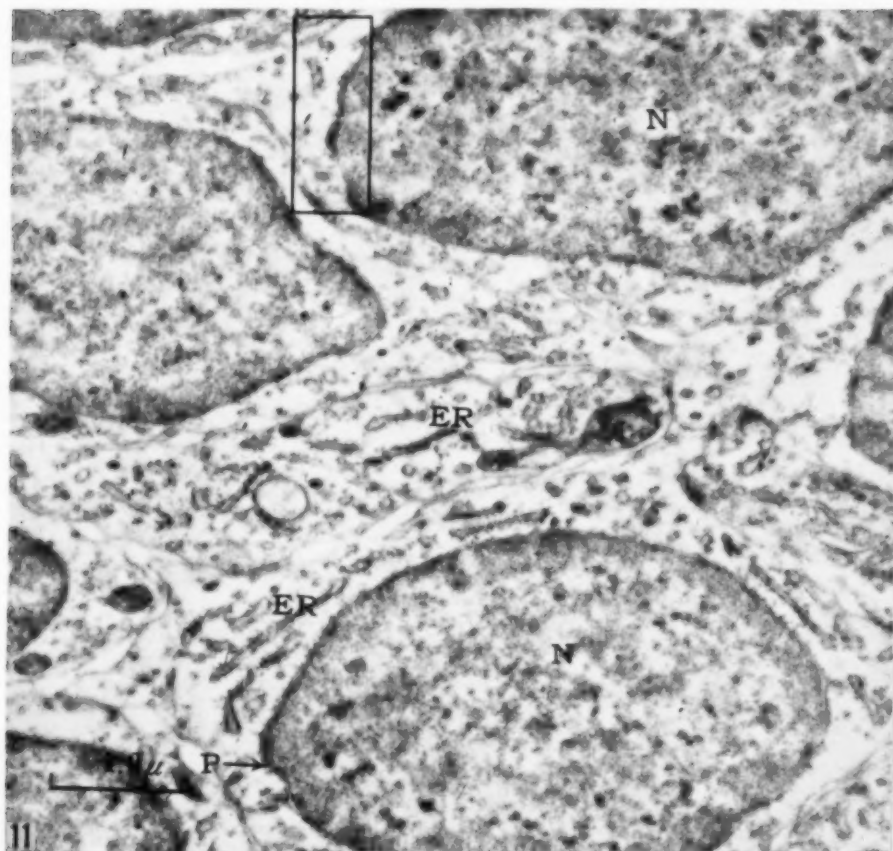
FIG. 11. Nuclei of ameloblasts (N). Small particles (P) can be seen on surface of outer nuclear membrane. The rectangle shows the area which was enlarged in Fig. 12. ×19,000.

FIG. 12. Part of nucleus (N) showing nuclear membranes (NM) and small particles (P). ×63,000.

PLATE 7

PLATE 8
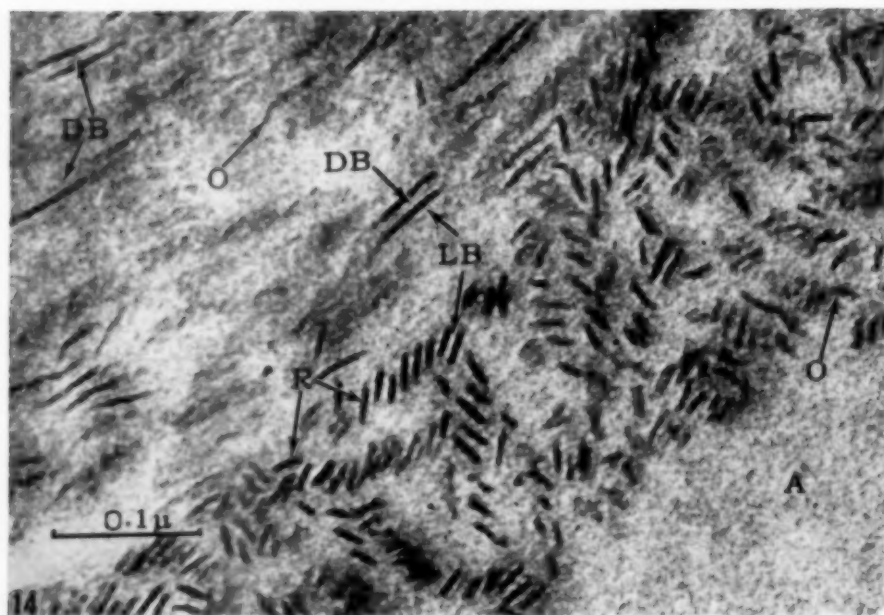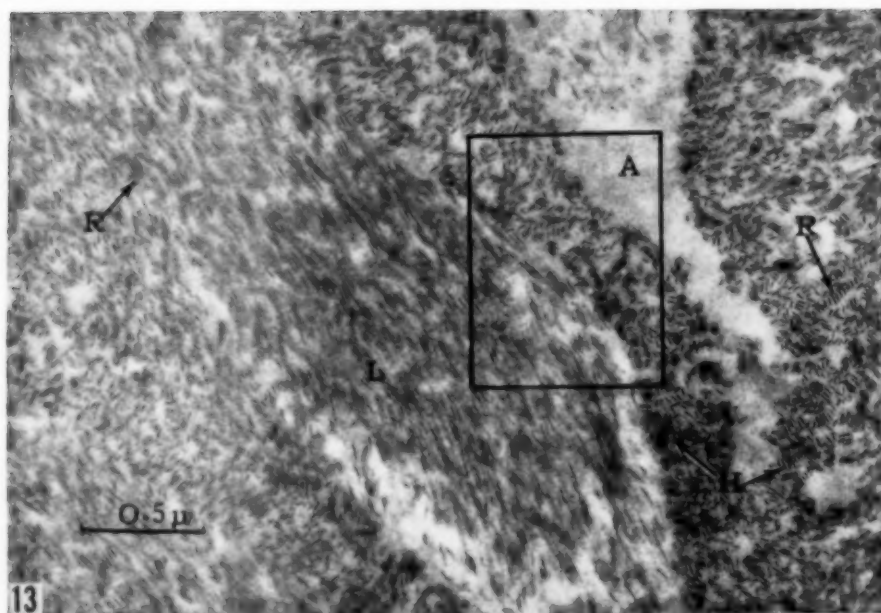
Fɪɢ. 13. Enamel matrix showing rodlets (R) and amorphous material (A). Some rodlets are oriented with their long axes in the same direction (L), others exhibit a "herringbone" arrangement (H). Rectangle |shows area which was enlarged in Fig. 14.   ×36,000.

Fɪɢ. 14. High magnification of enamel matrix. A light band (LB) is present along the sides of some rodlets (R). Amorphous material (A) is finely dispersed where there are no rodlets. Elsewhere, this material has varying degrees of opacity, some of which (O) may condense into forms which have the same shape as rodlets. It may also condense to form a dark band (DB) next to the light bands.   ×170,000.

Fig. 15.   Transverse section of ameloblasts (A) taken from more incisal region (arrow 1 of Fig. 1).   Note large amount of extracellular space (ES) and microvilli (MV).   ×12,000.

Fig. 16.   High power view of ameloblasts from most incisal region (arrow 2 of Fig. 1).   Note small 70 Å granules (G), desmosomes (D) and extracellular space (ES). ×70,000.

PLATE 9
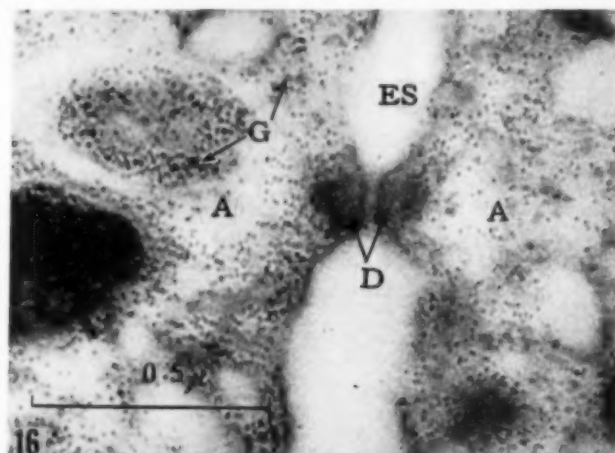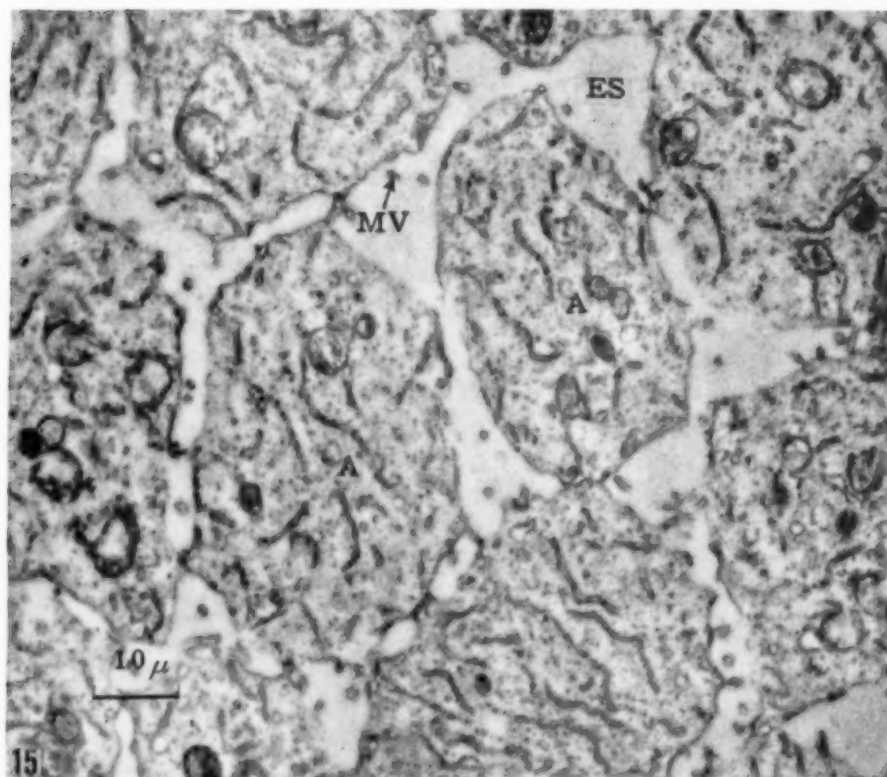
THE NEURO-HISTOLOGY OF MAMMALIAN BONE

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Abstract—The neuro-histology of bone was studied using a silver impregnation technique
and the reliability of the methods was examined quantitatively. A representative
selection of lamellar, woven and Sharpey fibre bone from rodent, monkey and human
specimens was studied. The observations confirmed the findings of some previous
workers that a rich plexus of nerves could be demonstrated in the periosteum, that
bone receives its innervation via nutrient canals and that nerve fibres ultimately reach
the Haversian systems. The observations of DE CASTRO (1930) that nerve fibres
terminate in or on the cytoplasm of the osteoblasts or osteocytes, could not be
confirmed, nor could any evidence be found to support HURRELL's (1937) view that
nerve fibres terminate within the bone matrix.

LE GROS CLARK (1958), discussing the current views on the neuro-histology of
bone, refers to the work of DE CASTRO (1930) and HURRELL (1937), and comments on
their view that adult bony tissue contains a number of nerve fibres, some ending blindly
in the bone matrix, others terminating in close relationship to bone cells. Most of the
published work on the neuro-histology of bone is based on the examination of sections
prepared by block impregnation methods. It seemed worthwhile therefore to re-
examine the subject using an alternative method of silver impregnation. FEARNHEAD
(1957) using a modified HOLMES (1943) technique, was consistently able to demon-
strate fine nerve fibres within the tubules in calcified dentine. As the problem of study-
ing the innervation of bone is comparable with that of demonstrating nerves in the
dentine, the method employed by FEARNHEAD (1957) was also used for this study.

MATERIALS AND METHODS

Bone from foetal and adult mice and rats, bone from adult rhesus monkeys and
bone from human foetuses was studied. All the material was fixed in 10% neutral
formal saline, with the exception of the monkey bone which was fixed in Bouin's
solution. Decalcification was achieved using a sodium formate–formic acid solution
(KRISTENSON, 1948) and the endpoint of decalcification determined radiographically
(MILES, 1949). The majority of the specimens were double-embedded in paraffin wax
celloidin, while some were embedded only in celloidin. Paraffin wax sections were
cut at a thickness of 10 μ and celloidin embedded material was cut at 15 μ. All the
material was cut in series and impregnated with silver. The silver technique employed
involves the control of temperature, pH and ionic silver concentration during the
initial impregnation of the tissue with the metal (FEARNHEAD and LINDER, 1956).
The principal stages in this technique are listed in Table 1.

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Table 1. Technique (after Fearnhead and Linder, 1956)

Principal stages	Notes
1. Wash deparaffinized sections.	2 hr in running tap water for formalin fixed tissues; 16–18 hr in running tap water for other fixatives, Bouin, Rossman, etc.
2. Three changes of distilled water.	1 hr each change—the last change in double glass-distilled water.
3. Overnight for 12–14 hr in concentrated Palitzsch boric acid borax buffer pH 7·0 maintained at 37°C.	Mix twice amount required, use half for initial buffering and remainder for silver impregnation.
4. 2 hr in 1:10,000 silver nitrate in Palitzsch buffer pH 7·0 maintained at 37°C.	When transferring to silver impregnating solution, agitate sections to remove bubbles which may otherwise adhere to section and interfere with the impregnation.
5. Rinse quickly in double-distilled water.	
6. Place in 2·5% sodium sulphite for 5 min at room temperature 20°C.	⎡ Mix the developer at this point and maintain at 29C° in a water-bath for the next 14 min. ⎤
7. Wash in running tap water for 4 min.	
8. Distilled water for 10 min.	Three changes, double glass-distilled water.
9. Developer for 3 min at 29°C.	
10. Wash in distilled water.	Until all trace of developing solution is removed.
11. Tone in 0·2% gold chloride for 10 min.	
12. Wash in distilled water for 1 min.	
13. Reduce gold with 1:2500 resorcinol in distilled water for 5 min.	The time taken for the reduction of the gold may be decreased by using a stronger solution of resorcinol.
14. Wash in distilled water.	
15. Place in 5% sodium thiosulphate for 5 min.	
16. Wash, dehydrate, clear and mount.	

In the silver impregnated material the whole area of bone present in at least fifty sections from each block was examined carefully under $\frac{1}{8}$ in. and $\frac{1}{12}$ in. (oil immersion) objectives. The fields consisted of a representative selection of woven bone (Baker, 1950) and lamellar bone. The bone surfaces included those from regions of tendinous insertions of muscles; Sharpey fibre bone, e.g. adjacent to the periodontal membrane; the outer surface of bone covered with periosteum but free of ligamentous insertions; the medullary surface of bone, and the interior of Haversian systems (Table 2).

TABLE 2. THE NUMBER OF SECTIONS EXAMINED (TOTAL, 4500) AND
THE SOURCE OF EACH BONE SAMPLE

Source of material	Maxilla	Mandible	Temporo-mandibular joint	Calvaria	Ribs	Limb bones
Adult mouse (*Mus musculus*)	300	450	600	100	100	—
Rat (*Rattus norvegicus*)	200	200	200	100	—	100
Adult monkey						
(*Cercocebus fulinginosus*)	—	200	—	—	—	—
(*Cercopithecus sabaeus*)	—	400	—	—	—	—
(*Macaca radiata*)	—	200	—	—	—	—
(*Macaca mulatta*)	—	200	—	—	—	—
Human, foetal } 92 mm ♂ / 28 week ♂	300	500	350	—	—	—

Reliability of method of silver staining

Although a consistent impregnation of the nerve fibres in calcified dentine, using this silver method, has been demonstrated (FEARNHEAD, 1957), it was considered necessary to establish on a quantitative basis the reliability of the technique in the case of bone. Ribs and intercostal regions of a mouse were embedded in paraffin and 10 μ serial sections cut transverse to the axis of the rib. The mounted sections were divided into batches, and each batch of sections impregnated with silver on a different occasion; any section which was not perfectly flat was rejected. Photomicrographs of a small nerve bundle were taken throughout the series, and subsequently enlarged to whole plate size. Forty photomicrographs representative of each batch were selected at random throughout the series and the number of the nerve fibres in the same nerve

TABLE 3. THE NUMBER OF NERVE FIBRES COUNTED IN SERIAL SECTIONS OF
AN INTERCOSTAL NERVE BUNDLE OF A MOUSE

Section	No. of nerve fibres	Section	No. of nerve fibres	Section	No. of nerve fibres	Section	No. of nerve fibres
2	183	14	180	26	181	43	172
4	172	15	181	27	183	47	182
5	175	16	183	28	175	49	171
7	173	17	182	29	176	50	187
8	179	18	180	30	181	53	187
9	181	19	183	33	175	56	186
10	177	20	173	37	184	57	189
11	179	20	181	38	175	58	183
12	177	23	183	39	175	59	181
13	173	24	175	41	175	60	183

Nerve fibres counted: maximum = 187; minimum = 171; average = 179. Standard deviation = 4·65

bundle was counted; the average number of fibres being 179 ± 8, giving a standard deviation of 4·65 (Table 3). On the basis of this analysis it was decided that the silver impregnation method demonstrated nerve fibres with a consistency satisfactory for this study. The variation in the number of fibres counted could be explained by nerve fibres leaving the nerve bundle, and by an increase in the number of fibres by division within the nerve bundle.

Morphology of nerve endings

In view of the controversial opinions concerning the morphology of nerve endings in close relationship to bone, it was necessary to define precisely what configuration of silver impregnated fibre would be acceptable as a true nerve ending. WEDDELL, PALMER and PALLIE (1955) reviewed the subject of the morphology of sensory nerve endings in mammalian skin. They have shown that it is characteristic of all nerves entering this integument to terminate in an arborization of fine, naked axoplasmic filaments which, in all probability, end freely. They also describe numerous encapsulated nerve endings in glabrous skin and mucous membranes, such as those of the lip, anus and glans penis. These nerve endings are of different sizes and shapes and the ensheathed myelinated stem fibres which enter the capsule then pursue a tortuous course. These fibres, in all cases, give rise to an arborization of fine naked axoplasmic filaments which end freely among the capsular cells in planes roughly parallel to the surface layer of cells.

In the light of these observations, it seems that both in encapsulated and in non-encapsulated endings the nerve fibres terminate in naked axon filaments which may appear beaded. It has been shown (FEARNHEAD, 1957) that very small beaded nerve fibrils are present in the dentinal tubules of human teeth. These fibrils are similar to the nerve endings described in skin, in that they possess no supporting Schwann cells for the terminal part of their course. They branch frequently before entering the dentinal tubules, and are approximately $0·2 \mu$ in diameter.

Thus, it seemed reasonable to suppose that nerves ending in or close to bone would have a similar morphology. In this study, therefore, both arborizations of naked axon filaments and encapsulated endings were sought. Nerve fibres were not regarded as terminating or being near their terminations unless they possessed the characteristic morphology described above.

RESULTS

Examination of serial sections through representative regions of all the specimens examined (Table 3) failed to confirm the view that nerve fibres enter the bone to terminate within the bone matrix.

A total of 4500 sections of bone was examined and in no case was a nerve fibre found to terminate in the bone matrix. In the Haversian canals the nerve fibres maintain a close relationship with blood vessels, either as single fibres intimately associated with vessel walls, or as small nerve bundles which accompany the vessels. No one example of an organized nerve terminal of any type was found within the Haversian canals or the periosteum. It is of interest, however, that the two fields illus-

trated (Figs. 5 and 6) show structures closely resembling encapsulated endings. Both of these fields lay close to the surface of the bone where it was cut prior to fixation, and must be regarded as artifacts caused by retraction of the nerve during the preparation of the material. Nerve fibres were found entering the nutrient canals and Haversian systems in company with vessels, and these fibres maintained a close association with the vessels throughout their course. Numerous terminal nerve fibres were found ramifying within the periosteum of the bone; the plexiform arrangement of these fibres being richer in areas of tendinous insertion, for example in the periodontal membrane and in the capsular region of the mandibular condyle (Fig. 9). Care was also taken to look at the cells of the periosteum for evidence of special nerve endings such as "*boutons termineaux*" (DE CASTRO, 1930) but no examples of this type of ending were observed.

DISCUSSION

It is difficult to accept a negative finding as conclusive, but it is considered that the method of silver impregnation used for this study has been shown to be reasonably consistent, even when examined quantitatively. Furthermore, as a large proportion of the material also included teeth in which terminal filaments were clearly impregnated within the tubules in the calcified dentine, it would seem reasonable to assume that any similar nerves which might be present in the bone would also be impregnated.

HURRELL (1937) claimed to have demonstrated nerve fibres terminating in the bone matrix. His results were based on one piece of tissue taken from a cat and stained by the block impregnation method of de Castro. The fibres illustrated by Hurrell as nerve endings, do not conform with the morphological criteria used for the identification of nerve endings in this study. They do, however, correspond with the morphology of large calibre nerve fibres. It is impossible to see from his photomicrographs whether or not Schwann cells are associated with these fibres although in his Fig. 2 a large slipper-shaped swelling associated with one of the fibres could well represent the position occupied by a Schwann cell nucleus (Fig. 4). According to our calculation the diameter of the fibre he illustrated varies from $1 \cdot 3 \mu$ to $0 \cdot 6 \mu$; thus, it was larger than any of the fibres of the nerve terminations described in this work. It is recognized, however, that such criticism on the basis of size may not be strictly valid, since it is not certain to what extent the silver impregnation has contributed to the apparent diameter of the fibres.

The advantage of clarity resulting from a minimal background silver impregnation of the bone matrix, achieved in successful de Castro preparations, has the disadvantage of not showing where the fibres are situated in relation to the surrounding tissue. In such preparations it is impossible to tell with certainty whether silver impregnated fibres are lying on the bone surface or in small bony canals rather than terminating in the matrix. This is a matter which can only be settled by continually changing the focus of the microscope whilst examining the sections, and it is important that the details of the background can be identified at the same time (Figs. 7 and 8).

DE CASTRO (1930) is of the opinion that nerve fibres in the periosteum end either in the cytoplasm or on the surface of the osteoblasts. This observation could not be confirmed, but it must be remembered that it is never possible to be sure that the

ultimate ending of the terminal filament is being viewed. For example, the plane of section may pass through a terminal nerve just proximal to its termination, the severed portion being too small for it to be identified in the next section of the series. Alternatively, terminal nerve fibres may gradually become narrower to a point where they can just be resolved by the optical microscope. It is possible, therefore, that these fibres continue to decrease in diameter, reaching sub-microscopic dimensions. The observations of DE ROBERTIS and SOTELO (1953) lend some support to this idea, since they have found, with the aid of the electron microscope, sub-microscopic extensions of axoplasm at the growing tips of neurones cultured *in vitro*.

It is conceivable that nerves in the periosteum lying close to bone surfaces may become trapped in the bone matrix during phases of bone remodelling. This phenomenon is known to occur quite commonly in the predentine of teeth (BRADLAW, 1939), although the fate of such loops is not known. However, since they are rarely found more than 4 or 5 μ from the calcification front of the dentine, it seems likely that such fibres become cut off from their nutrition and degenerate, perhaps leaving behind narrow canals as evidence of their position. This, however, is speculative, since no such canals have been demonstrated in dentine. The view that nerves may become enclosed in forming bone was suggested by DE CASTRO (1930). We were able to demonstrate nerve fibres close to the zone of ossification in growing bone (Figs. 1, 2, 3) but nerve loops comparable with those in predentine (Fig. 10) could not be found in the osteoid of the bone examined in this study. Making allowance for the difficulties of interpretation, and the limitation inherent in all silver impregnation methods, we are forced to conclude from the examination of our material that nerves do not end within the calcified bone matrix. We have also been unable to find any conclusive evidence of the existence of organized encapsulated endings, or "*boutons termineaux*" in bone.

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Figs. 1, 2 and 3. Fine nerve fibres lying near to a vascular canal in the zone of endochrondral ossification at the head of the mandibular condyle in a 28-week human foetus. Figs. 1 and 3 are high power views of two portions of the field shown in Fig. 2.

Fig. 4. An illustration from Hurrell (1937) for which the caption is "End of a nerve fibre in the bone matrix. Shows the histological characters, varicosity and beading A little below the middle of the left side of the field is the outline of a bone lacuna to which the nerve fibre ends are going." Magnification of original reproduction ×1500. Here reproduced with additional ×1·8 enlargement.

Fig. 5. Nerves lying in a bony canal having the appearance of an encapsulated ending. The cut surface of the bone is indicated by arrows. The complex morphology of this nerve fibre is attributed to retraction of the fibre from the cut surface and must therefore be regarded as a preparation artifact.

Fig. 6. A high power photomicrograph of a complex arrangement of nerve fibres similar to that shown in Fig. 5, taken from a similar location and also regarded as a preparation artifact.

Fig. 7. A tangential section of the wall of a bony canal which would appear to show a nerve fibre situated in the bone matrix, were it not for adequate background staining.

Fig. 8. A small bundle of nerve fibres passing through a bony canal.

Fig. 9. Fine terminal nerve fibres lying in the periosteum at the neck of the mandibular condyle. (Macaca mulatta).

Fig. 10. A nerve fibre passing from the pulp of a tooth into the predentine and looping back towards the pulp.
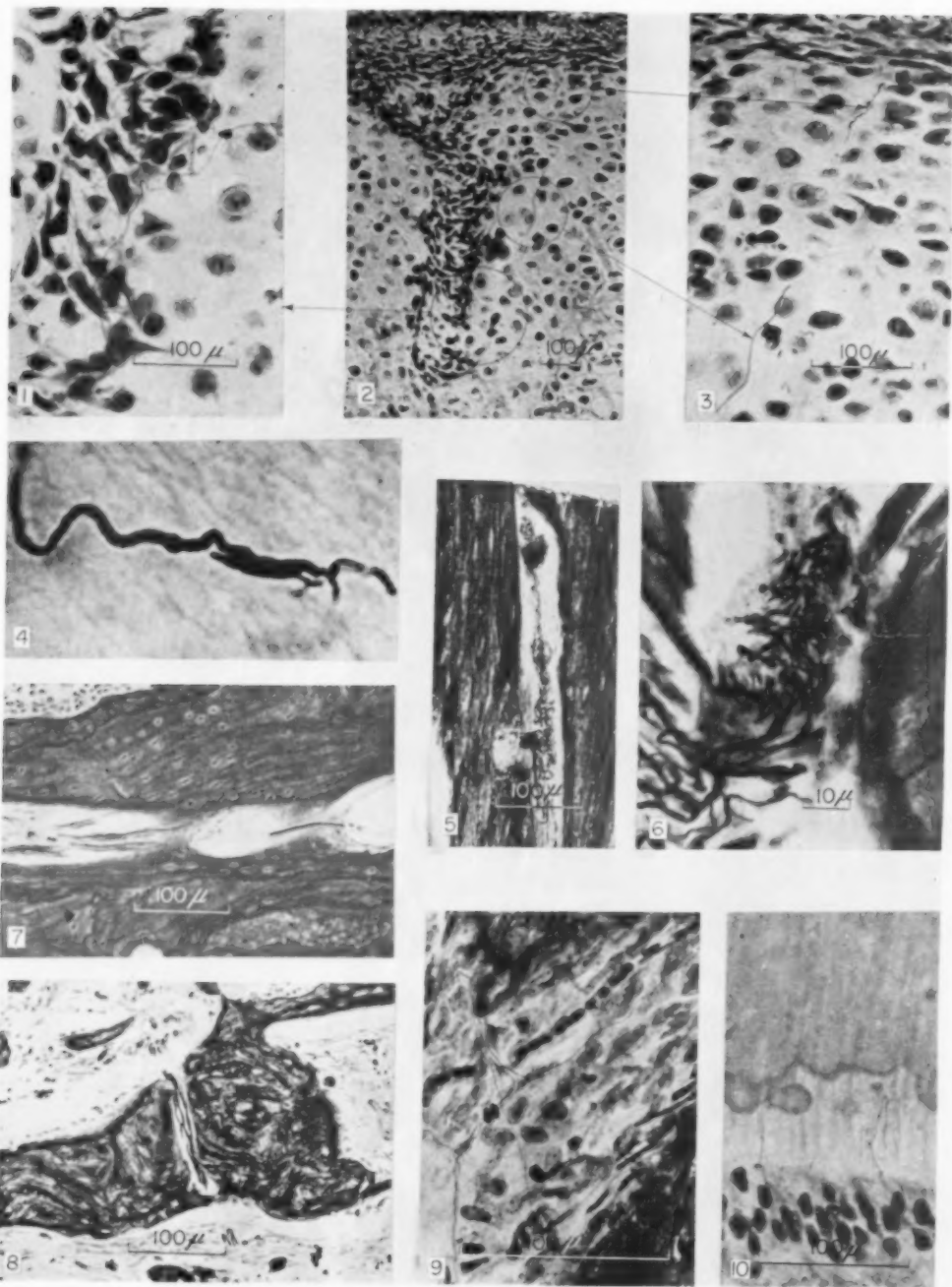
PLATE 1

HISTOCHEMISTRY OF THE GINGIVA—III

THE DISTRIBUTION OF AMINO-PEPTIDASE IN NORMAL AND INFLAMMATORY CONDITIONS

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Abstract—The distribution of a proteolytic enzyme (aminopeptidase) in gingival tissue has been ascertained using two different histochemical methods. Its distribution in the connective tissue fibroblasts, endothelial cells of blood vessels and in the stratum germinativum and spinosum of the gingival epithelium increases in inflammatory conditions. Lymphocytes and granulocytes contain a large amount of enzyme. The possibility that the increased enzymatic activity might play a role in the denaturation of proteins linked to highly polymerized carbohydrates is discussed.

THE distribution of acid phosphatase, beta-D-galactosidase, beta-glucuronidase, total nonspecific esterases, cholinesterase and succinic dehydrogenase in the gingiva has already been reported (QUINTARELLI, 1959; QUINTARELLI and LISANTI, 1959). The present investigation was undertaken to ascertain, if possible, the presence and localization of a proteolytic enzyme, an amino-peptidase, in clinically normal and inflamed gingiva.

Aminopeptidase is largely distributed in living tissues and organs (WHITE, HANDLER, SMITH and STETTEN, 1959). However, the presence of this enzyme could be demonstrated intracellularly only after FOLK and BURSTONE (1955) synthesized L-leucyl-beta-naphtylamide-hydrochloride, a substrate which permitted both the colorimetric visualization of aminopeptidase as well as the development of a histochemical method for its demonstration (BURSTONE and FOLK, 1956). Almost simultaneously, GREEN, TSOU, BRESSLER and SELIGMAN (1955) colorimetrically determined the same enzyme by an independent dye-coupling procedure. NACHLAS, CRAWFORD and SELIGMAN (1957) then established a histochemical technique using the latter technique on fresh frozen sections.

MATERIAL AND METHODS

This study was carried out on fourteen gingival specimens obtained from two groups of patients. Clinically normal gingiva was taken from seven people requiring tooth extraction and inflamed specimens were removed from seven patients who needed partial or complete gingivectomy for periodontal treatment. Immediately after removal specimens were briefly washed in a cold solution of 0·9% NaCl, to rid them of organic debris and blood, and cut into blocks of 2 or 3 mm in thickness.

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The method devised by BURSTONE and FOLK (1956) utilized the freezing–drying apparatus, which renders the sections ready for incubation without the use of any fixative, and with no apparent loss of enzyme activity. Therefore, some of the tissue blocks were immediately frozen at −70°C using dry ice and acetone. Since the method devised by NACHLAS, CRAWFORD and SELIGMAN (1957) permits fixation of tissues containing high aminopeptidase activity for 4 hr, samples were also fixed in neutral buffered formalin, or in a chloral hydrate–formalin mixture at −4°C (BAKER, HEW and FISHMAN, 1958), and from these routine frozen sections were cut.

Preparation of the substrate solution

Simultaneous coupling method (BURSTONE and FOLK, 1956). Two substrate solutions were employed. The first utilized 1 ml of a 1% stock solution of L-leucyl-beta-naphtylamide hydrochloride (Dajac Laboratories, The Borden Co., 5000 Langdon St., Philadelphia 24, Pa.) diluted in 40 ml of distilled water. To this solution 10 ml of a 0·2 M phosphate buffer, pH 6·9, and 30 mg of Garnet GBC (Imperial Chemical Industries Ltd., Manchester, England) were added, the mixture shaken and filtered into a coplin jar. The second substrate, i.e. the alanyl-beta-naphtylamide hydrochloride was used as recommended by the original investigator (BURSTONE, 1957). The stock solution was made up by dissolving 130 mg of this substrate in 3 ml of methyl alcohol. Following this, 500 ml of water were added, and the mixture was heated until complete solution was effected. To 40 ml of this solution, 10 ml of phosphate buffer pH 6·9 and Garnet GBC, in the proportions as described above, were added. Sections were incubated for 1, 2, 3, 4, 5, 12 and 15 hr at room temperature. They were washed in tap water and, without counterstain, were mounted on slides with glychrogel.

Post-coupling method (NACHLAS, CRAWFORD and SELIGMAN, 1957). To 2 ml of a stock solution (16 ml of leucyl- or alanyl-compound in 2 ml of water) 20 ml of a 0·1 M acetate buffer solution, pH 6·5, and 16 ml of 0·85% sodium chloride were added. Finally, 2 ml of 0·02 M potassium cyanide and 20 mg of tetrazotized diortho-anisidine (Diazo Blue B) were added to the mixture. The substrate solution was then poured into a coplin jar and the sections, mounted on slides, were incubated at 37°C for 1, 2, 3 and 4 hr. After incubation the sections were rinsed for 2 min in 0·85% saline and then placed in a solution of 0·1 M copper sulphate for 2 min. After a final rinse in saline, they were carried through various concentrations of alcohols, cleared in xylol and mounted in Permount.

Control

Rat kidneys were cut into small blocks and processed with the experimental tissues. Thus, some specimens were immersed in the different fixative solutions while others were cut in a fresh frozen state.

RESULTS

The sites of enzymatic activity, using BURSTONE's method, stained in different shades of red, whereas utilizing the technique suggested by NACHLAS, CRAWFORD and SELIGMAN (1957) the enzyme yielded a deep purple colour at the sites of high

activity, or various shades of orange, from intense to pale, in the less reactive structures. However, the pattern of enzyme concentration and distribution revealed by the two methods was the same.

The method of BURSTONE and FOLK could not be utilized in fixed frozen sections because the diazonium salt employed (Garnet GBC) crystallized in a few minutes. Such findings were observed even in rat kidneys, which were used as controls and were known to contain large amounts of the enzyme. With the technique suggested by NACHLAS, CRAWFORD and SELIGMAN (1957) fixed rat kidneys gave a beautiful reaction along the juxta-medullary portion of the cortex. Sections of gingiva fixed for 4 hr in either neutral buffered formalin or in chloral hydrate–formalin mixture failed to give a substantive reaction suggesting that the enzyme concentration in clinically normal gingiva was lower than in rat kidneys. The first attempts to obtain positive results with frozen specimens cut in their fresh state were unsuccessful. The possible explanation of these failures may be that the tissue, in its frozen state, was cut with the knife kept at room temperature. The difference in temperature between the frozen tissue ($-35°C$) and the knife ($22°C$) could have accounted for the enzyme inhibition. When a dry-ice carrier was laid on the knife, thereby decreasing its temperature to -4 or $-5°C$, the reaction was always positive.

The description of the cytologic distribution of amino-peptidase in human gingiva which follows is based upon the method of NACHLAS, CRAWFORD and SELIGMAN (1957), since the permanency of this reaction allowed observation and photography of the sections even after several months had elapsed, and was not complicated by crystallization artifacts.

The distribution of amino-peptidase in the gingiva differs according to the tissue structures involved. The highest concentration of enzyme was detectable in the fibroblasts. Their colour was uniformly deep purple, indicative of the greatest activity. The endothelial cells of the blood vessels also reacted very strongly while the loose connective tissue fibres showed somewhat less reactivity. A positive reaction was observed in the erythrocytes although to a lesser degree.

The gingival epithelium revealed a different colour intensity corresponding to the different strata. The stratum germinativum stained dark orange-purple thus indicating a fairly abundant amount of enzyme. The colour slowly diminished in the stratum spinosum and was even less in the parakeratotic layer (Fig. 1). Different reactions were noted in cases of progressive inflammation. In tissue affected by mild gingivitis, the intensity of the reaction was definitely greater, indicating an increase of amino-peptidase in the basal cells. These were stained much darker than in the so-called normal cases. A greater intensity of reaction was noted in the fibroblasts of the underlying connective tissue. The polymorphonuclear leukocytes in the diseased tissue gave a strongly positive reaction (Fig. 2). With both methods, but especially using the post-coupling technique, these inflammatory cells showed the most intense enzyme reaction, their colour being dark purple. The connective tissue, oedematous and disrupted in certain areas, also stained a deep purple. In cases of acute gingivitis with intense leukocytic infiltration, the amount of proteolytic enzyme was striking, and in these cases the stratified gingival epithelium also reacted intensely.

The stratum germinativum and the deepest cells of the stratum spinosum were both so highly reactive that their colour matched that of the leukocytes, thus indicating an unusually elevated proteolytic activity (Fig. 3). The gingival structures which normally are stained yellowish by this method, changed to a dark violet along the stratum germinativum and spinosum. However, in the sites where the inflammation was not particularly severe, the epithelium did not present the same colour intensity. The endothelial cells of the capillaries were also quite reactive.

Thus, it was concluded that in clinically normal conditions aminopeptidase in the gingiva was concentrated in the fibroblasts and in the endothelial cells of the blood vessels. The germinal layer showed an intense reaction while little enzyme activity was noticed in the stratum spinosum, and in the parakeratotic and keratotic layers. In the areas of acute inflammation the enzyme reaction increased progressively in all the gingival structures. The granulocytes always showed a high concentration of the enzyme and in certain cases of exacerbation of the inflammatory process, the connective tissue reacted intensely. In these instances drastic changes in colour were also observed in the stratum germinativum and stratum spinosum. The proteolytic activity of the epithelial cells equalled that observed in the connective tissue (Fig. 4).

DISCUSSION

There is little information about the proteolytic (proteinase and peptidase) activity in human gingiva. CHAUNCEY and QUINTARELLI (1959) presented information about the proteolytic activity of saliva and the possible relationship with periodontal disease. CHAUNCEY (unpublished) observed that among seventy-six micro-organisms found in the whole saliva, sixteen were able to elaborate proteolytic enzymes. Organism identification indicated that eleven were streptococci of the viridans group, four were *Pseudomonas aeruginosa*, and one was a *Staphylococcus citreus*. BURSTONE (1957b) carried out a comparative histochemical study of the proteolytic activity of human neoplasms and of inflammatory tissues. He believed that the highest aminopeptidase activity was to be found in the stroma of the tumour, and that the intense activity displayed by the stroma depended upon the invasiveness of the tumour. BURSTONE's findings were later supported by BRAUN-FALCO (1957) who demonstrated the same enzymatic characteristics in tumours and in inflammatory reactions of the skin. MONIS, NACHLAS and SELIGMAN (1959), on the basis of their histochemical investigations of neoplastic and inflammatory tissues, also demonstrated a general increase in proteolytic activity in the involved cells. These authors, however, stated that the increase in aminopeptidase noticed in the stroma of tumours is indicative of an enhanced fibroblastic activity rather than of an increased invasiveness of the neoplastic cells. SYLVÉN and MALMGREN (1957) put forward the hypothesis that the high content of proteases in tumour cells may determine the degree of destruction of the surrounding tissue. The findings reported in this paper demonstrate that the proteolytic activity in gingival tissue increases according to the degree of inflammation. Although the present data is exclusively histochemical, many papers give biochemical information regarding the proteolytic activity of different tissues (SMITH, 1948).

The proteases are enzymes which are capable of hydrolysing the peptide bonds of simple peptides or proteins. Enzymes similar to the leucyl- or alanyl-aminopeptidase are widely distributed in the tissues of animals (IRVING, FRUTON and BERGMANN, 1942; JOHNSON and BERGER, 1942) as well as in many micro-organisms (BERGER, JOHNSON and PETERSON, 1938; WEIL and KOCHALATY, 1937). Leucyl-peptidase has been found in a number of aerobic bacteria. Such bacterial enzymes appeared to be very similar in enzymatic properties to leucyl-peptidases from other sources. The possible explanation of the functional behaviour of proteolytic enzymes in different structural conditions is by no means defined. In inflammatory states the impaired blood supply and the subsequent shift in the local pH of tissue fluids to the acid side of neutrality, resulting from increased production of lactic acid by gly-colysis, would seem to favour proteolysis (RONDONI, 1951; RONDONI and POZZI, 1933; IRVING, FRUTON and BERGMANN, 1942). Furthermore, proteolysis of the tissue affected is enhanced by the increased occurrence of inflammatory cells. Leukocytes contain considerable dipeptidase activity (BARNES, 1940) and also cathepsins and other proteinases (FLEISHER, 1955).

No definite explanation has yet been found as to the possible mechanism exerted by proteolytic enzymes in tissues. Although proteolytic enzymes take a direct part in the destruction of tissue, the chemistry of this destruction is little understood. The extremely high protease content in the inflammatory cells is noteworthy. The possibility that granular leukocytes may liberate certain amounts of proteolytic enzymes is suggested for the following reasons:

In inflammation, cell autolysis and death occur as a normal physiopathological response to tissue injury. WAGNER and EHRICH (1950), on the basis of their studies of the adenosine deaminase content in lymphocytes, suggested that when autolysis occurs the cells liberate their nuclear products into the surrounding tissue. Studies on metachromasia carried out in gingiva revealed striking reactivity around the inflammatory cells, thus suggesting that leukocytes play a chemical role in the depolymerization phenomena rather than a simple mechanical disruption of the tissue cells or fibres (QUINTARELLI, 1960). Although no definite evidence has so far been brought forward one should not overlook the possibility that from these inflammatory cells there might be some type of "leakage" of enzymes into the extracellular spaces. On this basis, the possibility that cellular as well as bacterial proteolytic enzymes may play a role in the depolymerization of extracellular ground substance has been investigated and reported separately (QUINTARELLI, 1960).

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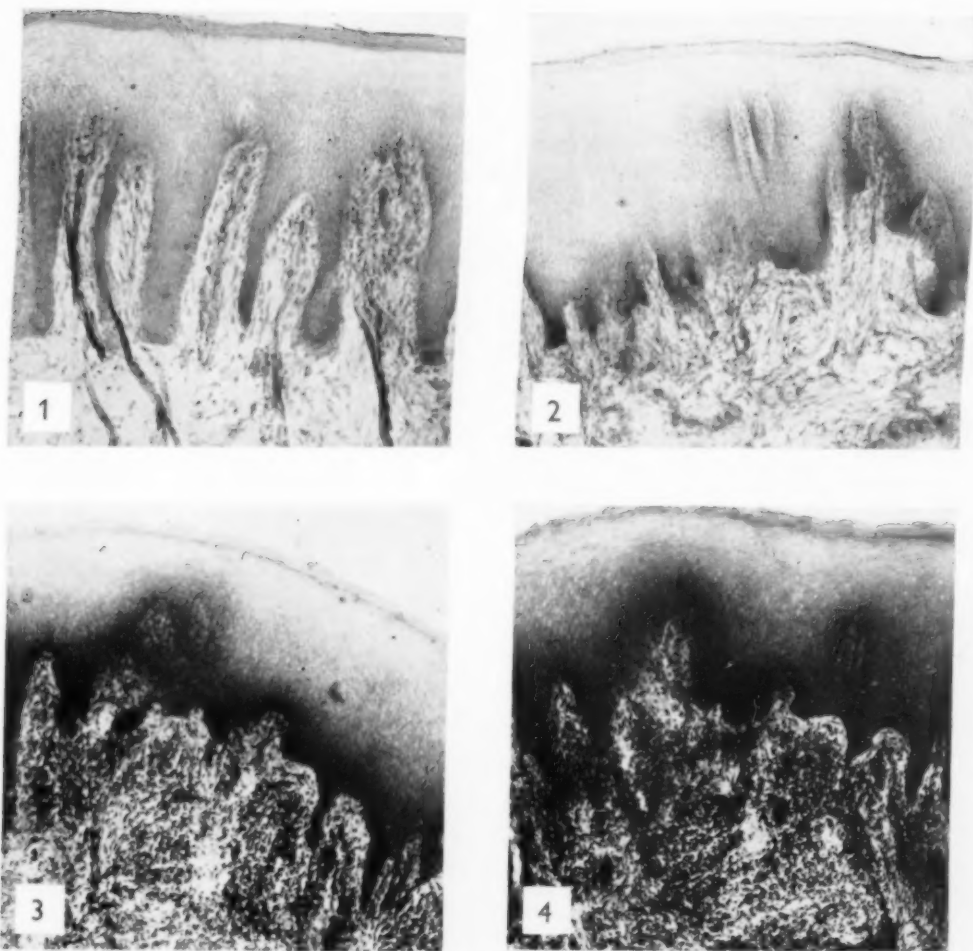
Figs. 1–4. Leucyl substrate. (Method of Nachlas, Crawford and Seligman, 1957). ×120.

Fig. 1. Moderate inflammation detectable in the interpapillary connective tissue. Activity is seen in the inflammatory cells, stratum germinativum, blood vessels and fibroblasts.

Fig. 2. The increased staining is observed in the connective tissue cells and stratum germinativum.

Fig. 3. The enzymatic activity is enhanced in the stratum basale and partially in the stratum spinosum. Connective tissue shows a striking increase in aminopeptidase mainly localized in leukocytes as well as in the fibroblasts.

Fig. 4. Further evidence of the enzyme distribution in a case of severe gingival inflammation.

Plate 1

HISTOCHEMISTRY OF THE GINGIVA—IV

PRELIMINARY INVESTIGATIONS ON THE MUCOPOLYSACCHARIDES OF CONNECTIVE TISSUE

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Abstract—The metachromatic phenomenon in the gingival connective tissue was studied. Clinically normal gingiva showed slight metachromasia. However, in inflammation the chromotropic material increased. The change in colour was greatest in the ground substance of the connective tissue in close proximity to the alveolar bone and in the areas infiltrated by inflammatory cells. Since connective tissue metachromasia may indicate the presence of acid mucopolysaccharides, and because of their increase in inflammatory conditions an effort was made to explain this phenomenon. Incubation of tissue sections with proteolytic enzymes showed a definite enhancement in metachromatic staining. Since proteolysis takes part in the degradation phenomena of the ground substance, this lends support to previous studies of the distribution of a proteolytic enzyme, aminopeptidase, in the gingiva under normal and inflammatory conditions.

THE histochemical determination of certain mucopolysaccharides in connective tissue ground substance has been long achieved by means of basic dyes. Of the forty-one dyes which have been studied (KELLY, 1956), toluidine blue has been, and still is, the most widely used. When tissue sections are stained with this cationic dye, some structures are coloured in different shades of violet which differ from the original blue. EHRLICH (1879) called this phenomenon metachromasia. No agreement has been achieved as to the interpretation of this metachromasia; however, it is generally agreed upon that polysaccharides of high molecular weight may be responsible for the peculiar change in colour. MEYER and RAPPORT (1950) isolated several acid mucopolysaccharides from different tissues of mesenchymal origin. They classified these highly polymerized carbohydrates as follows: (1) hyaluronic acid, which is sulphate-free and is rapidly digested by testicular as well as bacterial hyaluronidase; (2) chondroitin sulphate A, as the main component of hyaline cartilage, hydrolysed by testicular but not bacterial hyaluronidase; (3) chondroitin sulphate B (MEYER and CHAFFEE, 1941) isolated from pig skin, calf skin, tendon and aorta, resistant to the action of both enzymes; (4) chondroitin sulphate C which was observed in umbilical cords, heart valves and aorta, (this mucopolysaccharide is very similar to chondroitin sulphate A) and (5) chondroitin, obtained only from corneas. Basic dyes, especially those which produce metachromasia, are generally used in the histochemical study of mucopolysaccharides. The specificity of the reaction seems to depend upon the combination of the dye with the acid groups of the substrate molecules. Metachromatic substances may, in certain cases, be completely removed by hyaluronidase

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in tissue containing either hyaluronic acid or chondroitin sulphuric acid A and C (e.g. hyaline cartilage). Acid mucopolysaccharides may be found in tissue linked to proteins thus forming a continuous chain of carbohydrate protein bonds. Among the several substances which may influence metachromasia, proteins have the most striking inhibitory effect. This appears to be due to the fact that the acid groups of the polysaccharides, responsible for the metachromatic reaction, are blocked in their combined form. KELLY (1955) observed *in vitro* the blocking effect by protamine on the metachromatic reaction. The same dye-protein competition was also observed by FRENCH and BENDITT (1953) in tissue sections. However, when trypsin was utilized in both tissue sections and solutions prior to staining, metachromasia was once again detected (KELLY, 1951). Certain tissues in physiologic conditions show a faint metachromatic reaction, whereas, in diseased states they present a well defined metachromasia. In this regard WALTON and RICKETTS (1954) have recently postulated that increased metachromasia may be due to: (1) increased concentration of the acidic polysaccharides; (2) increase in the number of acidic groups; or (3) their unblocking by dissociation of a protein mucopolysaccharide complex.

In a previous paper (QUINTARELLI, 1960) the presence of a proteolytic enzyme, an aminopeptidase, was demonstrated histochemically in gingival tissue and it was also shown that there was a marked increase of this enzyme in inflammation. It was then thought that much increased proteolysis may play a certain role in the depolymerization of some components of the ground substance. These findings prompted an investigation into the effects of proteolysis in the mucopolysaccharides of the gingival ground substance.

MATERIALS AND METHODS

Biopsies of gingiva from patients in need of tooth extractions, human skin from the deltoid region of cadavers with no history or signs of dermatoses, and nasal cartilage of newborn rats were obtained and treated in the following manner: specimens were fixed in Carnoy, alcohol–formalin, neutral buffered formalin and 100% alcohol at 4°C for 48 hr. Specimens were then dehydrated in 100% alcohol, cleared and embedded in paraffin, and sectioned at 7 μ, deparaffinized and hydrated to 70% alcohol.

Staining solution

Toluidine blue O (American Aniline Co. C.N. No. 641) and azure A (American Aniline Co. C.N. No. 442) were utilized. Toluidine blue was used at concentrations of 0·2, 0·5 and 0·05% with M/100 citrate buffer (LILLIE, 1954) at pH 3·6, 4·3 and 7·0 and filtered through Whatman paper No. 1. Azure A was prepared at the same concentrations as toluidine blue, diluted in 70% alcohol solution and filtered. To both mixtures a few crystals of thymol were added as a preservative. Azure A was employed mainly because toluidine blue O in its commercial preparation is an admixture of pure and impure materials. KRAMER and WINDRUM (1955), using spectrophotometric studies, revealed five fractions of the dye, one of which consisted of a

blue fraction with a violet tint, and another with traces of a pale pink colour. Examination of the azure A revealed only three fractions, all having a blue hue. The staining periods varied according to the concentration of the dye. Therefore, sections were stained for 1 hr in 0·05% concentration of azure A and toluidine blue and for a 20 min period in 0·2 and 0·5% concentration. Because of the well-known troublesome effect of dehydration, sections were first studied under water; then they were dehydrated for 2 min in 95 and 100% ethanol (1 min each), cleared in several changes of xylene, mounted in Permount (Fisher Lab. No. 12-568) and examined. No aqueous mounting was employed since the dyes used dissolve very readily in aqueous media.

Enzyme solutions

Bovine testicular hyaluronidase (Wyett Institute for Medical Research, batches Nos. 96F02 and W380), papain (crude extract; Nutritional Biochemical Co. batch No. 1380), ficin (Worthington Biochem. Corp. batch No. 5803 B) and crystalline pepsin (Nutritional Biochemical Co. batch No. 9043) were used. Hyaluronidase slices incubated for 10 and 30 min, 1 and 2 hr. Papain and ficin were activated by incubating at 37°C for 30 min in 10 ml of 0·02 M acetate buffer, pH 5·4, which contained KCN (or cysteine) and EDTA in a concentration of 5 μM. Sections were then incubated for $\frac{1}{2}$, 1 and 2 hr at 37°C. Pepsin was made up in a solution of 0·02 N HCl at pH 1·6 containing 2 mg/ml of crystalline pepsin; sections were incubated for 10 and 30 min, 1, 2 and 6 hr. Controls have been used utilizing representative sections of the tissues studied and incubated in buffer solutions without enzymes for the same periods of time.

FINDINGS

The fixatives did not differ in their effect on the staining and all were considered satisfactory. However, Carnoy and alcohol–formalin (90 volumes of 95% ethanol and 10 volumes of 40% formaldehyde Baker-Lot No. 91297) seemed to preserve

TABLE 1. TOLUIDINE BLUE O

| Material | Water | | Intensity of metachromatic reactivity |
	Concentration (%)	pH	
Gingiva	0·2	3·6	
		4·3	Intense (bright pink)
	0·5	7·0	
Skin	0·2	3·6	
		4·3	Slight (blue-pink)
	0·5	7·0	
Cartilage	0·2	3·6	
		4·3	Very intense (reddish)
	0·5	7·0	

better cytological detail. No difference was ever observed in staining intensity and metachromatic reaction in sections stained at dye concentrations of 0·2 and 0·5%, used for the above suggested times. Tables 1 and 2 represent the intensity of the metachromatic reactivity of sections in toluidine blue and azure A, examined under water at different concentrations and pH. Table 3 shows the same observations in tissues dehydrated and mounted in Permount.

TABLE 2. AZURE A

| Material | Water | | Intensity of metachromatic reactivity |
	Concentration (%)	pH	
Gingiva	0·2	3·6	
		4·3	Moderate (light pink)
	0·5	7·0	
Skin	0·2	3·6	
		4·3	Slight (blue-pink)
	0·5	7·0	
Cartilage	0·2	3·6	
		4·3	Intense (bright pink)
	0·5	7·0	

As shown in Tables 1 and 2 the greatest shift in colour was noticed in the cartilage and the lowest in the skin. Gingival tissue also gave rise to metachromatic reaction at the various pH values. However, with toluidine blue the metachromatic intensity was stronger than with the azure A. Almost all the gingival tissue examined showed different degrees of inflammation. Such differences helped in differentiating the more reactive areas from the less reactive ones. In general, it may be said that the most reactive areas were around the inflammatory cells. In certain cases of lymphomono-cytic infiltration the shift in colour was definitely stronger than in other tissue areas.

Of great interest was the reaction of the connective tissue adjacent to the alveolar bone. In many instances of chronic gingivitis, the heavy deposits of chromotropic material were observed along the line where the connective tissue adheres to the alveolar bone. The interpapillary connective tissue was less reactive (light pink) especially when free from leukocytic infiltration. In cases where necrotic foci were found there was no metachromasia. The mast cells showed the greatest reactivity while the intercellular material of the endothelium of the small arterioles and capillaries also demonstrated a definite change in colour. In a few cases in which mild inflammation was present in the deepest connective tissue, the intercellular ground substance showed a faint pink colour. In other instances a number of connective tissue papillae in the neighbourhood of the attached gingiva were orthochromatic, while others, especially those located in the upper portion of the free gingiva were often reactive.

TABLE 3. TOLUIDINE BLUE O OR AZURE A

Material	Water		Intensity of metachromatic reactivity
	Concen-tration (%)	pH	
Gingiva	0·2	3·6	Moderate (light pink)
		4·3	
	0·5	7·0	
Skin	0·2	3·6	Negative
		4·3	
	0·5	7·0	
Cartilage	0·2	3·6	Intense (bright pink)
		4·3	
	0·5	7·0	

As can readily be seen after alcohol dehydration the metachromatic phenomenon is completely abolished in the skin, is much decreased in the gingiva and is still present, although to a lesser degree, in the cartilage matrix. Untreated and digested sections were stained in azure A and toluidine blue O in solution at pH 4·3. They were then washed in distilled water, dehydrated and mounted. Table 4 describes the staining reactions after enzyme digestion. No difference was observed in regard to the different concentrations of the dyes used at the stated times.

TABLE 4

Enzyme	Material	Azure A	Toluidine blue O
Pepsin	Gingiva	Bright pink	Bright pink
	Skin	Negative	Negative
	Cartilage	Reddish	Reddish
Papain	Gingiva	Bright pink	Bright pink
	Skin	Negative	Negative
	Cartilage	Digested	Digested
Ficin	Gingiva	Bright pink	Bright pink
	Skin	Negative	Negative
	Cartilage	Digested	Digested
Hyaluronidase	Gingiva	Blue	Blue
	Skin	Blue	Blue
	Cartilage	Blue	Blue
Control	Gingiva	Pale pink	Pale pink
	Skin	Blue	Blue
	Cartilage	Bright pink	Bright pink

Most of the pepsin-digested sections were completely lost during incubation or in the subsequent washing. A few sections, incubated for 20 min, could be utilized because, although the stratified squamous epithelium was almost entirely digested, the intrapapillary connective tissue remained. These papillae did not show any enhancement in metachromasia. The most reactive areas were in the deeper connective tissue invaded by inflammatory cells, in close proximity to the alveolar bone. However, with prolonged incubations of $1\frac{1}{2}$–2 hr there was an almost complete removal of collagen fibres. Sections pretreated with hyaluronidase were always orthochromatic with both toluidine blue O and azure A.

Although several sections of skin were treated as described above, only a few resisted enzyme digestion. However, the few slices of tissues which were examined did not reveal any enhancement in colour. These results differ from the findings of FOLLIS (1951) who reported that peptic as well as tryptic digestion of skin produces metachromatic reaction by unmasking previously bound sulphated mucopolysaccharides.

Altogether different was the reaction noted in the nasal cartilage. While pepsin enhanced the metachromatic reaction to a reddish pink hue, treatment with activated papain removed entirely the metachromatic colour so that the intercellular matrix appeared completely inactive and decolourized. Only the chondrocytes showed some scattered chromotropic granules.

DISCUSSION

In a previous paper (QUINTARELLI, 1959) an initial study of the metachromatic phenomena in gingival tissue was reported. While that work was in progress, there appeared a paper by FASSKE and MORGENROTH (1958) which gave information about the metachromatic reaction of the connective tissue ground substance of the gingiva. These authors associated the presence of chromotropic material with the different degrees of inflammation. The results reported here show that the metachromasia of gingival tissue increases proportionately with the intensity of the inflammatory phenomenon. Furthermore, in spite of a decreased shift in colour the metachromatic ground substance is alcohol resistant (see Table 3) and contains certain mucopolysaccharides unaffected by dehydration. Clinically normal gingiva, which on histological examination reveals little inflammation, contains very little chromotropic material.

The treatment of sections with proteolytic enzymes such as pepsin, ficin and papain enhanced the metachromatic phenomenon. This suggests that the anionic groups of mucopolysaccharides are masked by certain proteins. Digestion of proteins determines the liberation of the tissue anionic groups from their protein linkage. These anionic groups, in turn, are bound by the cationic groups of the dye. In so doing, the metachromatic phenomenon is greatly augmented. Gingival sections treated with hyaluronidase were always orthochromatic. Such a reaction indicates that chromotropic material in the sections might be either hyaluronic acid or chondroitin sulphate, this latter having been demonstrated to be bound to proteins (SHATTON

and SCHUBER, 1954). OGSTON and STANIER (1951, 1952) drew attention to the fact that hyaluronic acid was found in the synovial fluid as a carbohydrate–protein complex. Further evidence was given by BLUMBERG and OGSTON (1957a, b) who claimed that, after treatment of ox synovial fluid hyaluronic acid with papain, the proteolytic enzyme split the protein component and released the polysaccharide. Although the use of proteolytic enzymes to reveal acidic groups of mucopolysaccharides masked by proteins is quite common in biochemistry, it has been seldom employed in histochemical techniques. WINDRUM and KRAMER (1957), studying the histochemical reactions of amyloid, employed several proteolytic enzymes in an endeavour to demonstrate the masking of the carbohydrate moiety by proteins. Their results demonstrated that the action of proteolytic enzymes produces the metachromatic phenomenon in amyloid-affected ovaries, livers and kidneys which, in spite of evidence for the presence of sulphated polysaccharides (HASS, 1942), do not exhibit true metachromasia. As previously stated, FOLLIS (1951) occasionally observed a metachromatic reaction in skin sections pretreated with pepsin and trypsin, but in the present study it was not possible to confirm these findings. The same conclusions were reached by WINDRUM and KRAMER (1957) who could not reproduce FOLLIS' (1951) results. However, it should be emphasized that skin may give different reactions according to different anatomical regions. Unfortunately FOLLIS (1951) did not specify the anatomical region from which the fragments of epidermis had been removed. THOMAS (1956), injecting papain intravenously in young rabbits, noted a collapse of the ears that lasted 3–4 days. Histological examination revealed a complete loss of metachromasia and basophilia in cartilage matrix. The same results were obtained by BRYANT, LEDER and STETTEN (1958), using the same methods. These authors also noted an increase in mucopolysaccharides in blood and urine. Although no explanations have yet been put forward for this curious phenomenon, the authors assumed that the proteolytic action of papain causes a massive release of mucopolysaccharides from the cartilage matrix. This investigation indicates that the connective tissue ground substance of the gingiva contains metachromatic material resistant to dehydration in ethanol. This would seem to indicate that substances other than phosphoric groups are responsible for this phenomenon.

Inflamed gingival tissue is much more metachromatic than the normal tissue. The possibility that such increased chromotropism may be due to a releasing action of the protein component of mucopolysaccharides is further substantiated by the results of pretreatment of tissue with proteolytic enzymes. In fact, in cases where incubation with pepsin, papain or ficin was maintained for 30 min, a definite increase of metachromasia was noticed. Prolonged incubations for 1 or 2 hr, especially with pepsin, completely destroyed metachromasia and the collagen fibres were partially digested. Treatment of tissue sections with testicular hyaluronidase abolished metachromasia, thus suggesting that the chromotropic material was either a hyaluronate, a chondroitin sulphate or a combination of both. Because the metachromatic reaction appears simply as an indication of certain free anionic groups in the tissues and not, as is the general belief, as a specific histochemical identification of some chemical

groups (SYLVÉN, 1958), studies now in progress are aiming at establishing the types of mucopolysaccharides present in the gingival ground substance.

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ENAMEL FORMATION IN AMELOBLASTOMAS

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Abstract—Enamel formation has been observed in six of seventeen solid, cystic, acanthomatous or dentigerous (mural) ameloblastomas. Droplets of young, transitional, semi-mineralized or more fully mineralized enamel structures have been observed. Amelogenesis in ameloblastomas unrelated to dentine formation is similar to the findings of atypical amelogenesis in animal experiments. The presence of enamel droplets surrounded by ameloblasts in an ameloblastoma indicates that, as in certain other neoplasms, a high degree of differentiation may be achieved.

SEVERAL authors (D'AUNOY and ZOELLER, 1929; BERNAYS, 1885; CHIBRET, 1894; ROTTER and LAPP, 1958; SIEGMUND and WEBER, 1951) have suggested that formation of enamel, chiefly in the form of organic matrix, takes place in ameloblastomas. The dominant opinion, however, is that enamel formation does not occur in typical ameloblastomas (BERNIER, 1955; BOYLE, 1955; KRONFELD, 1930; ROBINSON, 1937; SCHMIDT, 1922; STONES, 1954; THOMA, 1954). Also, it was reported that no enamel formation occurred (ZEGARELLI, 1944) in a large series of "adamantinomas" from an inbred strain of mice.

It has been stated that enamel does not form in the absence of dentine (BUNTING and HILL, 1940; SCHMIDT, 1922) and that, therefore, ameloblastoma cells remain in an immature stage of development (KRONFELD, 1930; SONESSON, 1950) or that they bear only a superficial resemblance (STONES, 1954) to enamel-forming cells.

During a review of extensive material from our animal experiments (BOYLE, 1938; BOYLE and KALNINS, 1960; HOWE, WESSON, BOYLE and WOLBACH, 1940; KALNINS, 1952) the similarity of developmental disturbances of enamel formation (Figs. 1, 2, 5, 12) in the teeth of these animals to material found in a surgical biopsy specimen of ameloblastoma coming to us for histopathological diagnosis led us to examine other typical ameloblastomas with the purpose of ascertaining whether enamel formation was a more or less constant finding in this neoplasm.

MATERIAL AND METHODS

Seventeen solid, cystic, acanthomatous or dentigerous (mural) ameloblastomas were examined. When entire specimens were available, a large number of sections were prepared and were serially stained with haematoxylin–eosin, van Gieson, and Mallory's aniline blue connective tissue stain. The latter stain allows differentiation of young enamel matrix from transitional matrix (CHASE, 1935). Moreover, the use of these stains was essential for distinguishing droplets of enamel matrix from dystrophic calcifications, colloid drops and epithelial pearls. When found, such matrix formation was compared with disturbed amelogenesis in the constantly erupting teeth of rodents.

OBSERVATIONS

In six of the seventeen ameloblastomas enamel formation was observed. Since relatively few sections of several of the other ameloblastomas were available for examination it is conceivable that the actual incidence of enamel formation may have been higher. In the acanthomatous ameloblastoma illustrated in Figs. 3 and 4 large number of sections had been prepared but only in a few sections could droplets of enamel matrix be demonstrated.

Enamel droplets were found in epithelial islands where the stellate reticulum was undergoing squamous metaplasia (Fig. 4). With the Mallory stain some of these droplets appeared red, indicating the presence of young enamel matrix, others stained blue which is characteristic of transitional enamel matrix. This is essentially identical with findings in the teeth of rodents where enamel matrix is deposited not only upon the surface of essentially normal enamel but also as droplets dislocated into the interdental cartilage cementum of guinea pig molars (Fig. 5).

Apparently squamous metaplasia in the first instance and embedding into cartilage cementum in the second instance precluded further maturation of organic matrix to mineralized enamel.

A dentigerous ameloblastoma (Fig. 6) showed enamel formation in almost all of the sections. Young and transitional enamel matrix, together (Fig. 7) or separately, was found.

These droplets, in various stages of maturation, may be surrounded by radially oriented, columnar or cuboidal ameloblasts. In Fig. 8, a growing droplet of young enamel matrix (red staining—Mallory) has apparently been formed as the surrounding ameloblasts receded peripherally. In Fig. 10 the remnants of enamel matrix area in a transitional (blue staining—Mallory) stage at the periphery of the droplet while most of the central part of the droplet had become sufficiently mineralized for it to be dissolved during decalcification of the specimen. In Figs. 7 and 9 the ameloblasts toward the left appear functional and the adjacent matrix is in the young, red staining stage. On the right side the ameloblasts have atrophied and the adjacent matrix is transitional (Fig. 7) or the enamel has become partially acid soluble (Fig. 9). Some sections showed ossification and dystrophic calcification of degenerated ameloblasts and embedding of enamel matrix droplets in material resembling cementum or bone (Fig. 11). A similar pattern has also been observed in rodent teeth with disturbed amelogenesis (Fig. 12). This bone-like material stains blue in Mallory and red in van Gieson stains.

The remaining four cases also showed enamel formation in the form of enamel matrix or there were droplets of organic material surrounded by spaces which are suggestive of spaces formerly occupied by mineralized enamel (Figs. 13, 14).

DISCUSSION

Numerous observations from animal experiments in which disturbed amelogenesis has been produced show that ameloblasts detached from the underlying enamel matrix differentiate further and are capable of forming droplets of enamel matrix in the periodontium (BAUER, 1931; BECKS and FURUTA, 1939; CHASE, 1932; KALNINS,

1939; KOTANYI, 1927; SCHOUR and SMITH, 1934). These droplets later become mineralized (BOYLE and KALNINS, 1960; KALNINS, 1952a, b) (Fig. 2). That formation of enamel can take place independently of the presence of dentine was reported also in intraocular transplantation experiments of tooth germs (FLEMING, 1952).

Our findings are in accord with the results of animal experiments, since in typical ameloblastomas amelogenesis also occurs without the presence of dentine.

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Fig. 1. Acid resistant (organic matrix) enamel droplet (X) in the area of cemento–enamel junction in a guinea pig incisor with disturbed amelogenesis. P = periodontium. E = space formerly occupied by acid soluble enamel. D = dentine. C = cementum. Haematoxylin-eosin. ×300.

Fig. 2. Mineralized enamel droplets (indicated by arrows) in the periodontium (P) of a guinea pig tooth with disturbed amelogenesis. A ground section photographed in reflected light. E = enamel. D = dentine. ×45.

Fig. 3. Acanthomatous ameloblastoma. E = the lining of oral epithelium below which there are strands of epithelial cells. X = indicates the location of droplets of enamel matrix. Haematoxylin–eosin. ×12.

Fig. 4. One of the epithelial strands from the area marked X in the previous figure. The enamel droplet is surrounded by an area of squamous metaplasia of former stellate reticulum. Haematoxylin–eosin. ×500.
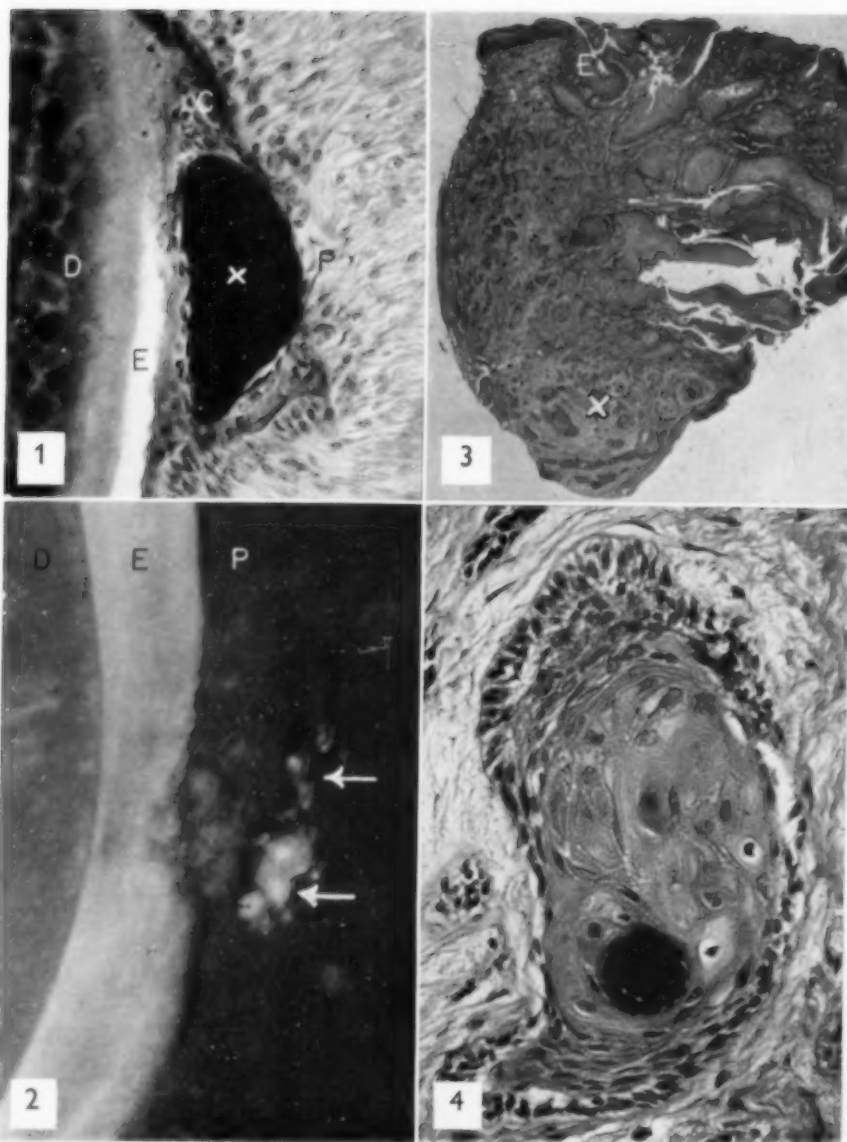
PLATE 1

PLATE 2
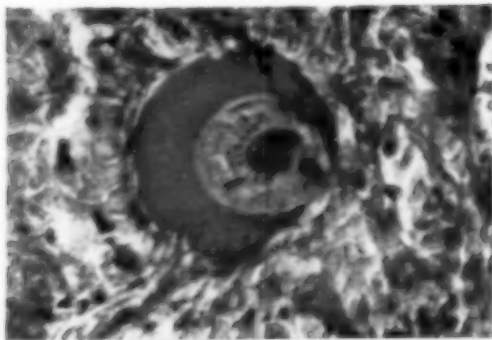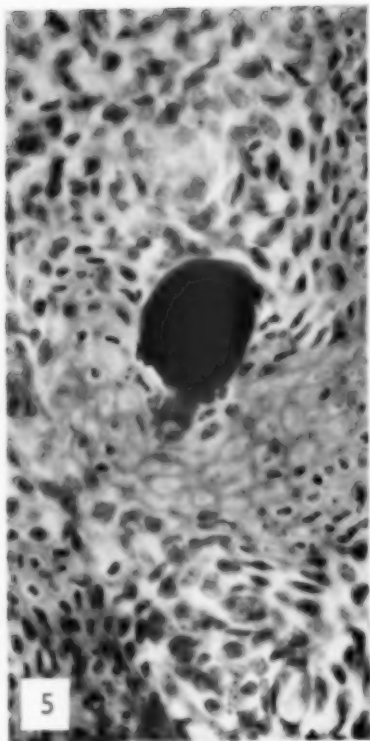
FIG. 5. Enamel matrix droplet in cartilage cementum of a guinea pig molar. Haematoxylin–eosin. ×500.

FIG. 6. Mural ameloblastoma arising from the wall of a dentigerous cyst. E = enamel (of the crown of the tooth) dissolved during decalcification in the course of histologic preparation. Haematoxylin–eosin. ×10.

FIG. 7. Enamel matrix droplet from the previous figure. The droplet is in the stage of maturation of young enamel matrix (red) into transitional matrix (blue). Mallory stain. Orig. magn. ×1000.

FIG. 8. An enamel droplet from Fig. 6. Adjacent to the droplet, radially oriented columnar cells indicate that this structure is not a dystrophic calcification but a growing enamel droplet. Van Gieson. ×450.

FIG. 9. Semi-mineralized enamel droplet from Fig. 6. Enamel matrix (x) is located between the active ameloblasts (on the left side of the picture) and the mineralized enamel; i.e. the space formerly occupied by mineralized enamel and partially dissolved by acids during histologic preparation. On the right side of the figure the ameloblasts adjacent to the mineralized portion of the droplet are atrophic. Haematoxylin–eosin. ×450.

FIG. 10. Mineralized droplet from Fig. 6. Radially oriented ameloblasts surround the droplet (i.e. the space formerly occupied by the acid soluble enamel). Remnants of the organic matrix appear at the periphery and toward the centre of the droplet. Haematoxylin–eosin. ×450.

FIG. 11. Enamel droplets with ossification, from Fig. 6. Ossification (indicated by arrows) and inclusion of the enamel matrix droplets (some of them indicated by x) in bony or cementum-like material. Haematoxylin–eosin. ×360.
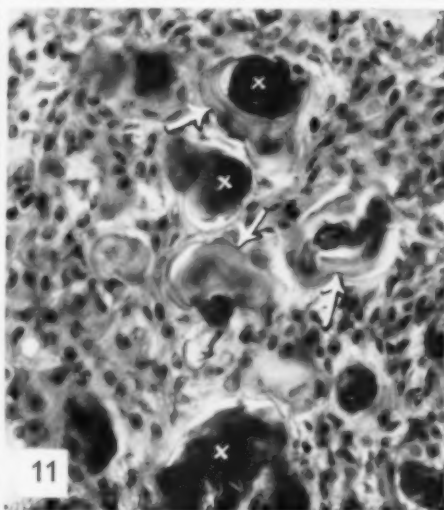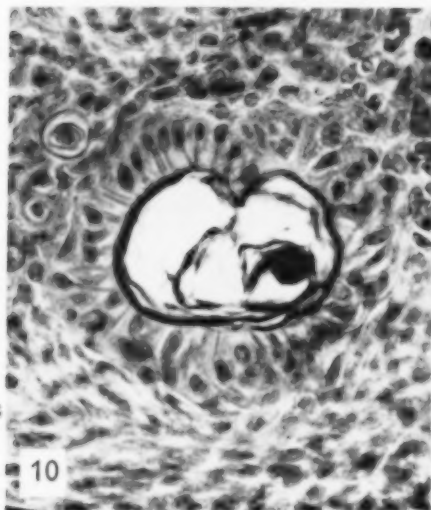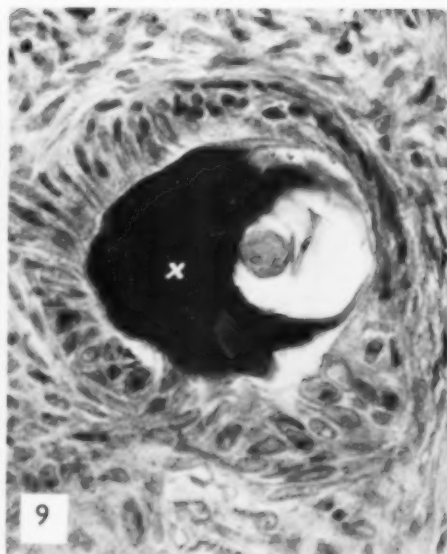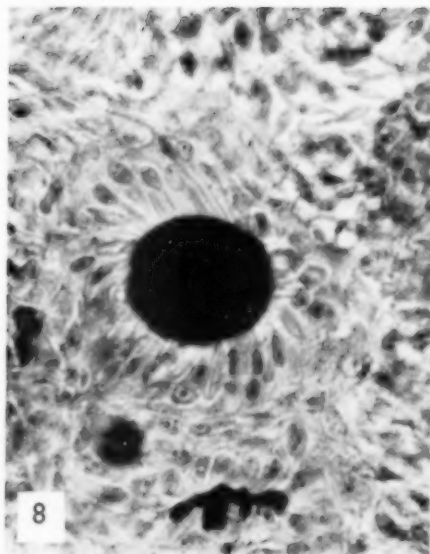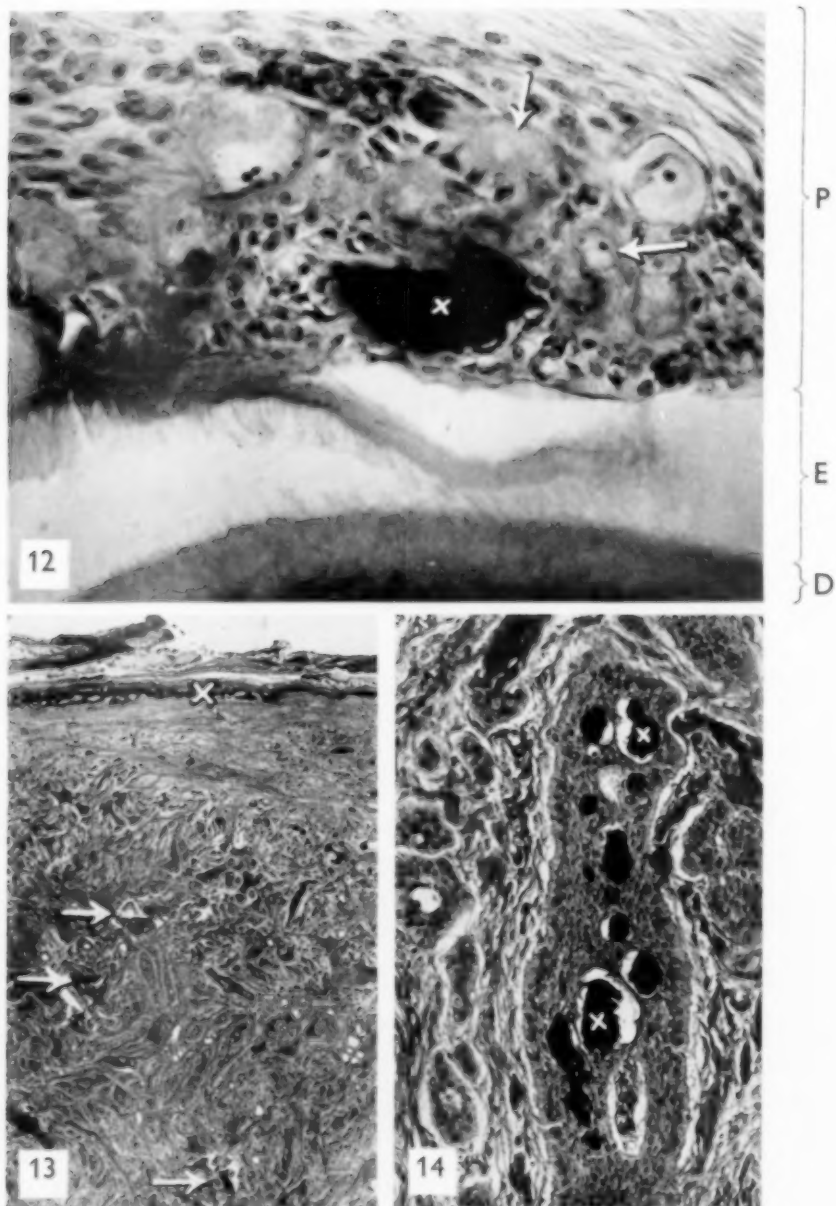
PLATE 3

PLATE 4

Fig. 12. Droplet of enamel matrix (x) surrounded by cementum-like material (arrows) in periodontium (P) of a guinea pig incisor. E = space formerly occupied by partially acid soluble enamel. D = dentine. Haematoxylin–eosin. ×400.

Fig. 13. Solid ameloblastoma. x = the bony capsule surrounding the neoplasm. Arrows indicate some of the sites where enamel droplets have been observed. Haematoxylin–eosin. ×20.

Fig. 14. Several enamel droplets (some indicated by x) in a strand of epithelium from one of the sites indicated by arrows in the previous figure. Haematoxylin–eosin. ×200.

DENTAL CARIES AND FLUOROSIS IN ISRAEL

A SAMPLE SURVEY ON ORAL HEALTH OF SCHOOL CHILDREN

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Abstract—A survey on oral health was conducted on a sample of about 4500 school children in Israel, by two dentists checked for the comparability of their methods of diagnosis, who performed all examinations, by means of mouth mirror and explorer, during a period of 10 weeks. Findings were expressed as DMFT for caries and in terms of Dean's classification for fluorosis. Percentages and averages were calculated for sub-groups according to sex, origin, sojourn in Israel and type of settlement and were compared. Caries prevalence was low, especially in oriental communities, and lower in new immigrants than in children born and reared in Israel. Children in collective settlements (kibbutsim) had significantly less caries experience than children in towns and villages. There were higher caries rates in girls than in boys, and in the mandible than in the maxilla. The relation between the percentage of children having at least one DMF tooth and the mean DMF count of a group differs in Israel from the formula given by Knutson, although there is a high degree of correlation between these two expressions of caries prevalence. The amount of dental restorative care, as expressed by the ratio F/DMF, is equal in towns and in kibbutsim, and considerably higher than in smallholders' settlements. Arab children do not receive any dental care.

Few children exhibited signs of fluorosis, most of them immigrants from countries where areas of endemic fluorosis have been observed.

INTRODUCTION

THERE is probably no country in the world whose population is so small and at the same time so heterogenous as that of Israel; about half of the present 2,000,000 inhabitants arrived after the establishment of the state in 1948. They came from over fifty different countries, including the whole continent of Europe, both Americas, Africa, the Near East, Yemen, India and China (STATISTICAL ABSTRACT, 1955/56). Among the old established population there are about 200,000 Arabs (STATISTICAL MONTHLY, 1956). Sixty per cent of the Jewish and about one third of the Arab population live in urban communities (ISRAEL YEAR BOOK, 1955/56); the rural settlements include the traditional village, smallholders' co-operatives (moshave ovdim) and collective settlements (kibbutzim). Thus there live in the country side by side communities differing widely racially, culturally, socially and economically, and as elsewhere it may be assumed that these factors strongly influence the oral health of the communities.

In order to assess the adequacy of existing dental care facilities and to plan improvements if necessary, the Ministry of Health became interested in gathering basic information on the oral health status of the different population groups. A number of investigations had been carried out in this field previously, especially regarding dental caries. MANSBACH (1937) was the first to carry out dental examinations among school

children; his results suggested striking differences in caries prevalence between the various types of settlements, but were not expressed in terms which would make his investigation reproducible. LAUFFER (1955) examined the students from Tel-Aviv schools, and WEINREB and BEN-SHUSHAN (1956) examined young male adults in the army. In 1953, following a suggestion made by Dr. Isaac Schour during his first visit to Israel, a survey was conducted by volunteers of the Israeli Dental Association under the auspices of the Ministry of Health. More than 11,000 school children aged 13–14 years were examined and the findings tabulated. The results were not published, but the data gathered seem to indicate that caries prevalence in Israel is considerably lower than in comparable population groups in Western countries, that it is higher in children of European origin than in Orientals and that the difference is highest among new immigrants and tends to level off the longer they live in the country.

All these investigations show a number of common weaknesses; the groups examined were not selected according to statistical principles of sample design, they did not include representative parts of all population elements, and the multiple examiners had not been tested to ensure that their assessments were comparable. Therefore the Ministry of Health decided to conduct a sample survey, which would allow valid generalizations for the population as a whole and could be reproduced in the future.

The present report includes the findings of caries prevalence. Since dental caries and fluorosis are interrelated public health problems the data on prevalence of fluorosis were added to this section of the survey.

COMPOSITION OF THE SAMPLE

Since caries is the main oral health problem and caries activity is highest in young adolescents (PELTON, 1955) it is customary to examine children after the exfoliation of the primary teeth, in order to measure caries experience in a population (KNUTSON, 1955). On the basis of the findings of the previous survey, which served as a

TABLE 1. COMPOSITION OF THE SAMPLE

Origin	Town				Village				Kibbutz				Total			
	1*	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Israel	152	0	0	152	18	0	0	18	44	0	0	44	214	0	0	214
Iraq–Iran	90	177	0	267	5	289	0	294	0	0	0	0	95	466	0	561
Yemen	20	45	0	65	20	231	2	253	42	9	0	51	82	285	2	369
Europe	341	1021	2	1364	119	348	0	467	87	28	0	115	547	1397	2	1946
Near East	15	73	1	89	0	53	1	54	8	5	0	13	23	131	2	156
North Africa	29	272	3	304	6	186	3	195	12	53	1	66	47	511	7	565
Arabs	102	0	0	102	485	0	0	485	0	0	0	0	587	0	0	587
Others and unknown	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	59
Total	749	1588	6	2343	653	1107	6	1766	193	95	1	289	1595	2790	13	4457

* 1 = In Israel before 1948; 2 = In Israel after 1948; 3 = Unknown; 4 = Total.

pilot study, and the experience gained in surveys elsewhere, it was estimated that a sample of 4500 children (about one eighth of the population of this age) would be sufficient to show any differences between subgroups of about 500 individuals (CHILTON, 1953; TREZONA, 1951; AMERICAN DENTAL ASSOCIATION, 1955). The criterion in designing the sample was the geographic origin of the children. Since it would have been impossible to draw groups from every country of immigration, states with similar ethnic and cultural patterns were combined into seven groups: "Near East", "Yemen", "North Africa", "Europe", "Iraq-Iran", "Arabs", and "Others and Unknown". Place of origin was arbitrarily determined as the country of birth of the father. This evidently is unsatisfactory in cases where the parents came from different countries but, since intermarriage between people of quite different origin is at present still rather exceptional in Israel (PERETZ, 1958), knowledge of the father's birthland provides adequate information for all practical purposes. Children who were born to fathers who were themselves indigenous to the country were assigned to a further group "Israel".

The "Near East" group includes Syria and Lebanon as well as Egypt. Like most Jewish communities, the immigrants of these countries are culturally very similar to their host nations, in this case the most progressive and "westernized" Arabs. In these states Jews lived exclusively in the major cities, and belonged largely to the middle classes as tradesmen, government officials, and members of the professions. In Yemen Jews lived under medieval conditions; when they were taken to Israel by plane most of them thought they were borne on "eagle's wings". They have their own very distinct and most traditional culture, primitive though delicate (COHEN, 1956; SHUVAL, 1955). Naturally, concepts of modern hygiene and health care were unknown to them, but they were keen to learn and proved to be the most adaptable of all oriental Jews (RIEGER, 1952; SHUVAL, 1955). Although there exist considerable differences among the Jews from Libya, Tunis, Algier and Morocco, the four countries which are included in the "North African" group, they have in common that they form a society in the state of transition from the traditional oriental to a modern pattern (BAR-YOSSEF, 1958).

A strong trend of migration from the villages of the Atlas mountains, where Jews generally lived as craftsmen and peddlers to the cities, is recognizable. They cram into the already over-crowded ghetto, the worst slums imaginable, and here they live mostly in poverty, lack of hygiene and insufficient nutrition. They feel strongly attracted by the French culture, but in most cases its influence remains quite superficial, and only a few succeed in ascending socially and economically. Naturally the urge to emigrate is strongest among the poor, and in fact most immigrants from these countries to Israel come from the villages and the lower social strata of the cities (ROSENFELD, 1958).

The group "Iraq and Iran" includes people not only from two countries, but also from three anthropologically different communities. There are first the Jews from Bagdad who, like many of the urban Persian Jews, are fairly well advanced in modernization and assimilation to the more progressive elements of their host-nations (ROSENFELD, 1957). Also there has been some movement of Jews in both directions across the frontier between Iraq and Iran, and therefore it was considered permissible to include them in a single group. In both countries there live members of another, quite distinct, community, the Kurdish Jews. Whereas the aforementioned speak Arabic, the language of the latter, besides Kurdish, is Aramic. Very much like the tribe of the Kurds they are typical mountaineers, a great many of them peasants. They are accustomed to hard physical labour. Standards of cleanliness and hygiene are very low, and learning and progress are not in high esteem (FEITELSON-SCHOR, 1958). Since countries had been the characteristic in determining origin it was impossible, in the framework of this survey, to isolate the Kurdish from the rest of the immigrants from Iraq and Iran. Therefore this part of the sample is less homogeneous than desirable, a fact which doubtless has some bearing on the results.

Most of the 200,000 Arabs of Israel live in villages, in their traditional way. Their sense of hygiene and their understanding of modern health practices, especially in the field of prevention, are little developed (ROSENFELD, 1956).

The differences between immigrants from various European countries, as well as those from America, were not thought important enough to justify a further subdivision.

Only children born in 1943 and 1944, therefore aged 13 or 14 years, were included. The sex distribution was about equal in all the ethnic groups. Information concerning the origin of the majority in the different schools had been obtained from the Ministry of Education and was supplemented by a postal questionnaire. On the basis of this knowledge all the children from different schools were classified into the seven groups, and in each group an appropriate number of children was randomly chosen. To ensure sufficient representation of the smaller population groups their share in the sample was increased.

STATISTICAL TREATMENT

As the ethnic structure of the population was known it was possible to calculate for each group its probability to be included in its specific group of the sample. Since the various ethnic groups are of quite different size a strictly random sample would be likely to include, for example, too small a number of Yemenites. Therefore, in planning the sample, care was taken to ensure that it included more Yemenites than their share in the whole population would actually justify. Thus, their probability for inclusion was higher than that of other children. These probabilities were calculated for each group and thus each observation received its own weight. These probabilities were recorded on the examination forms during the coding process. Since there are only very few entirely homogeneous school classes among the Jewish population, students of the same origin were found in different strata, possessing different probabilities. Thus every individual observation bears the weight of the group in which it was made. Accordingly, frequency distributions of findings for the different population groups were obtained of the individual scores (x_i)—e.g. DMF = 0—as weighed frequencies (f_i), giving instead of the total absolute number of observations a quantity ($\sum f_i$) expressing the weight of the group relative to the whole population. Arithmetical means were calculated according to the formula

$$\bar{X} = \frac{\sum f_i x_i}{\sum f_i}.$$

Similarly

$$\sigma = \sqrt{\left(\frac{\sum x_i^2 f_i}{\sum f_i} - \bar{X}^2\right)}$$

and

$$S.E. = \frac{\sigma}{\sqrt{n}}.$$

By these procedures population estimates were obtained, rather than the statistics derivable from the absolute sample values.

To isolate factors of environment influencing the developing teeth, the children were further divided into two groups: those living in the country before 1948, and those having arrived later, when they were already 5–6 years of age and the development of the crowns of their permanent teeth presumably was completed (SCHOUR and MASSLER, 1949). In order to investigate social factors the subjects were also classified according to length of residence and type of community.

Findings were expressed in commonly accepted epidemiologic indices: for caries experience the DMFT index was applied. Although less refined than the surface count (DUNNING, 1947) it is also less influenced by examiner's error; fluorosis was scored according to DEAN's classification (1934).

EXAMINATION AND RECORDING

Since examiners' errors are apt to impair the validity of surveys (AMERICAN DENTAL ASSOCIATION, 1955) great care was taken to restrict this possible source of misinformation. The whole survey was carried out by two examiners only, who were nearly of the same age and had received their professional education at the same University. Each examined in three of the six districts of the country. Before starting on the field work, they spent two days examining the same children. At first their findings differed considerably, primarily because one of them, having practiced preventive treatment for a number of years, was inclined to diagnose pits as carious lesions. Discrepancies were reconsidered and discussed, until finally uniformity of judgement was established. In order to reduce further the influence of personal opinion, it was decided to record teeth requiring extraction as "decayed". Examinations were carried out by daylight, in the classrooms, using mouth mirror and explorer. There is no doubt that, under these conditions, the overlooking of a number of cavities led to underestimation of caries prevalence, but it was the purpose of this survey to provide data on the relative caries experience and other oral morbidity statistics in various subpopulations, rather than to arrive at comprehensive diagnoses for treatment planning. Since it has been shown that a high correlation exists between carious lesions found clinically and the total number present (KNUTSON, 1955) lack of radiographs should not impair the validity of this investigation.

The examiners' findings were recorded—generally by the school nurses—on a specially designed form which was divided into three parts. The first part, which had been filled in by the class teachers prior to the examination, contained demographic characteristics of the student examined, district, town (village), school, name, sex, year and country of birth, year of immigration and father's country of birth. The second part of the chart contained the examiner's findings, and the third part was designed for statistical summaries, which had to be done during the coding process. The fact that all the observations had been registered in the form of indices made this work very simple. Finally, the coded items were transferred to punch cards and processed.

DIFFERENCES IN CARIES EXPERIENCE BETWEEN ETHNIC GROUPS

Table 2 shows the total caries experience of the children examined, according to their origin, expressed by mean DMF per child, and the variance. But since the distribution of DMF is far from normal, it is not properly described by mean and variance (CHILTON, 1953). Therefore the percentages of children without caries experience in each group have also been added, since DMF = 0 is the mode in all groups. When listed in an ascending order according to mean DMF, there appears a strong inverse correlation between these two statistics ($r = -0.97$).

TABLE 2. CARIES EXPERIENCE ACCORDING TO ORIGIN

Origin	Average DMF	Variance	% DMF = 0
Yemen	2·03	6·413	43·3
Arabs	2·26	6·341	39·1
Iraq–Iran	2·35	6·657	34·6
North Africa	2·38	6·882	38·9
Europe	2·83	7·399	26·6
Israel	2·95	9·357	26·5
Near East	2·97	6·622	21·4

When the means of the different groups are compared there are some which differ significantly from each other, whereas between others no statistically significant difference exists (Table 3). It appears that four groups—Yemen, Arabs, Iraq and North Africa—are similar regarding caries experience, whereas Europe, Israel and Near East belong to a different universe. The same relation concerning statistical difference between the rates of DMF = 0 is found. The group "others" has not been included in these considerations, because of its small size and its heterogenous composition. Thus the whole sample may be divided into two categories, one composed of the first four groups, which may be termed the oriental, and the remainder,

TABLE 3. COMPARISON OF MEAN DMF AND PERCENTAGE OF DMF = 0 BETWEEN GROUPS

Groups Compared	Diff. of Means	Sign	Diff. % DMF = 0	Sign
Yemen–Arabs	0·234	− −	4·2	− −
Yemen–Iraq	0·307	− −	8·7	− −
Yemen–N. Africa	0·332	− −	4·4	− −
Arabs–Iraq	0·073	− −	4·5	− −
Arabs–N. Africa	0·098	− −	0·2	− −
Iraq–N. Africa	0·025	− −	4·3	− −
Europe–Israel	0·075	− −	0·1	− −
Europe–N. East	0·183	− −	5·2	− −
Israel–N. East	0·098	− −	5·1	− −
Yemen–Europe	0·822	+ +	16·7	+ +
Yemen–Israel	0·897	+ +	16·8	+ +
Yemen–N. East	0·995	+ +	21·9	+ +
Arabs–Europe	0·588	+ +	12·5	+ +
Arabs–Israel	0·663	+ +	12·6	+ +
Arabs–N. East	0·861	+ +	17·7	+ +
Iraq–Europe	0·515	+ +	8·0	+ +
Iraq–Israel	0·590	+ +	8·1	+ −
Iraq–N. East	0·688	+ +	13·2	+ +
N. Africa–Europe	0·491	+ +	12·3	+ +
N. Africa–Israel	0·565	+ +	12·4	+ +
N. Africa–N. East	0·663	+ +	17·5	+ +

− −, P Value = 0·05 and more; + −, P Value = 0·01–0·05 (borderline significance);
+ +, P Value = 0·01 and less.

the occidental population. These terms are used in a cultural rather than geographical sense. The former is distinguished by a lower average of caries experience (2·03–2·38), and a higher percentage of individuals without caries experience at all (34·6–43·3), and differs in both respects statistically significant from the rest (Table 3).

Evidently the caries experience of Israel school children is lower than in most other populations about which comparable data have been reported. FINN (1952) tabulated fourteen surveys on school children, conducted mostly in the United States. The DMF rates of children 13 and 14 years of age vary between 3·66 and 8·55. MASSLER, PINDBORG and MOHAMMED (1954), in another compilation of epidemiologic studies include only surveys from India (SHOURIE, 1941), and Camden and Glynn counties in Georgia (HAGAN, 1947) with lower DMF values for this age group. According to TOVERUD (1956–57) the average DMF of children 12 and 13 years old in Norway were between 3·8 and 13·7. BARNARD (1956) reported the figures for Australian 13 and 14 year old children as 10·70 and 12·78. In the channel system suggested by MASSLER, PINDBORG and MOHAMMED (1954) evidently Israel would find its place for all population groups in the channel of "very mild" caries susceptibility.

It is possible that there exist different patterns of shedding and eruption of teeth between the various groups, which may be responsible to some extent for the differences in caries experience. No investigations in Israel have been published so far on this subject. Some observations seem to indicate differences in height and weight between population groups of different origin (STRAUSS, SHATAN-HERZBERG and BORTEN, 1954), but the available information is not sufficient to decide whether these differences are racially inherent or rather a consequence of social factors, which have their bearing on nurture and development.

The practice in mouth hygiene certainly varies considerably between children of differing origins, but no high correlation could be proved between this factor and caries experience (BARNARD, 1956; HEWAT, 1956; MASSLER and SAVARA, 1951). An inverse correlation between the annual amount of sunshine received and caries experience has been reported (HEWAT, 1956; MASSLER and LUDWICK, 1952); however, no satisfactory explanation for a causative relation has been offered. Therefore, the large amount of sunshine in Israel cannot explain the low caries experience.

Each group of immigrants to Israel brings with it its specific food habits, and tries to preserve them in the new environment. Nevertheless it was established in several surveys (STRAUSS, SHATAN-HERZBERG and BORTEN, 1954; STRAUSS, SHATAN-HERZBERG and RIM, 1955) that because of the rationing of certain foods a number of generalizations may be made. In families of comparable size (5–6 members) sugar consumption varies between 5·0 and 12·0 kg per month in families from Europe, between 4·7 and 8·5 and from 5·0 to 13·0 in different groups of oriental origin, whereas in Yemenite families the consumption was as low as from 1·5 to 8·0 kg per family. This shows an overall low consumption of the foodstuff which, according to present knowledge, plays the most important part in the aetiology of dental caries (SCHOUR and MASSLER, 1945). It was found that large amounts of bread are eaten by all population groups: 250 g daily in 1950, and 390 g in 1952/53; standard bread of 85 per cent extraction and enriched with calcium and riboflavine being 65·7 per cent

of the total dietary intake in Yemenites, 74·7 and 49·9 per cent in other "oriental" families and 52·6 per cent in European families. Generally it has been found that the oriental communities prefer spicy food, rich in fat and vegetables rather than sweets and snacks. About the Yemenite Jews the statement is made: "The ready availability of fresh foodstuffs direct from field to kitchen was a typical feature of the nutritional habits of Yemenite Jews, who came from small towns and villages", and again ". . . in spite of long residence in Israel, these families kept as far as they could, to their old ways. Preserved foods are completely rejected. Where possible, the traditional bread is baked at home. Usually, however, black bread is eaten" (STRAUSS, SHATAN-HERZBERG and BORTEN, 1954). It appears that food of all ethnic communities in Israel is poor in readily fermentable, cariogenic carbohydrates, and contains a sufficient amount of the essential nutrients. A further nutritional factor which has an important bearing on caries prevalence is the intake of fluorine, particularly during the period of tooth development. Little is known about the fluorine content of water supplies in most of the countries of origin of the oriental Jews, except for a few reports on endemic fluorosis in certain parts of the Atlas mountains (VELU, 1933), Yemen (CLAWSON, KHALIFAH and PERKS, 1940; EL TANNIR, 1959) and Turkey (ATA, 1956). Observations of fluorosis in the present survey are reported and analysed in one of the following sections. Surveys of the water resources of Israel carried out by GDALIA (1953) showed that water supplies contain between 0·2 and 1·5 p.p.m. fluorine, increasing from the North to the South. Owing to the fact that most places receive their supplies from various sources, with considerable seasonal fluctuations, an assessment of the influence of this factor is impossible. For this reason no attempt was made in the present survey to establish correlations between caries experience of children born and reared in different communities. Moreover, there is no reliable information on the optimal content of fluorine in the water supply for a subtropical country like Israel. According to a formula arrived at by GALAGAN (1957), this optimum should be around 0·7 p.p.m., but the influence of relative humidity, drinking habits and other pertinent factors are entirely unknown. Considering the fact that, besides water, the Israeli diet contains considerable quantities of foods rich in fluorine, such as tea and sea fish, it would appear that generally the fluorine intake of children in Israel is not far from the optimum, but this problem deserves further investigation.

DIFFERENCES IN CARIES PREVALENCE BETWEEN JAWS

In all groups a higher DMF rate was found for the mandible than for the maxilla, this difference being statistically significant for Arabs and the Near East group (Table 4). This is apparently in contradiction to most investigations as reviewed by FINN (1952), as well as to the findings in Israel by WEINREB and BEN-SHUSHAN (1956). However, it should be borne in mind that in a child population 13 and 14 years of age with such low DMF rates as those reported in the present survey the teeth affected are predominantly the first molars. It is well established that the lower first molars, probably for anatomical reasons, are more susceptible to caries than the upper molars (BEN-SHUSHAN, 1956; BARNARD, 1956; HEWAT, 1956; TOVERUD, 1956–57).

TABLE 4. CARIES EXPERIENCE: MAXILLA AND MANDIBLE

Origin	Maxilla DMF	Mandible DMF	Difference	Significance
Israel	1·38	1·57	0·19	−
Iraq–Iran	1·07	1·28	0·21	−
Yemen	0·97	1·05	0·08	−
Europe	1·26	1·57	0·31	−
Near East	1·20	1·77	0·57	+
North Africa	0·97	1·41	0·44	−
Arabs	0·85	1·51	0·66	+

FINN (1952), summarizes: "The great freedom from dental caries of the lower anterior teeth more than makes up for the greater susceptibility to dental caries of the lower first molars". Thus the higher caries rate observed in the mandible throughout this survey is in accordance with the findings in other investigations.

SEX DIFFERENCES

It was found that in all the ethnic groups included in this survey with the exception of the Iraq–Iran groups, caries experience was higher in girls than in boys. This excess was statistically significant in all cases (Table 5). This observation is in accordance with the findings of other investigators (BARNARD, 1956; HEWAT, 1956; TOVERUD, 1956–57), and is generally explained by the earlier eruption of permanent teeth in girls, causing these teeth to be a longer period at risk of becoming carious than in boys of the same age.

TABLE 5. CARIES EXPERIENCE—SEX SPECIFIC

Origin	Male DMF	Female DMF	Difference	Significance
Israel	2·296	3·016	0·720	+
Iraq–Iran	2·277	2·156	0·121	−
Yemen	1·450	1·805	0·355	+
Europe	2·660	3·092	0·432	+ −
Near East	2·014	3·275	1·261	+
North Africa	2·360	2·830	0·470	+
Arabs	2·083	2·793	0·710	+

+, critical ratio 3·0 and more; + −, critical ratio 2·0–2·5; −, critical ratio less than 2·0.

DIFFERENCES IN CARIES EXPERIENCE BETWEEN OLD-ESTABLISHED AND IMMIGRANT POPULATION

A breakdown of the diverse ethnic groups according to their period of living in Israel (Arabs and Jews of second generation in the country being excepted) shows that there are no significant differences in caries experience except in two groups: Yemenites and Europeans (Table 6). Whereas the former experienced an increase

TABLE 6. CARIES EXPERIENCE ACCORDING TO PERIOD OF RESIDENCE IN ISRAEL

Origin	Before 1948	After 1948	Difference	Significance of difference
Yemen	3·005	1·742	+1·258	+
Iraq–Iran	2·281	2·365	−0·084	−
North Africa	2·010	2·435	−0·425	−
Near East	3·204	2·925	+0·279	−
Europe	2·391	3·003	−0·612	+

of lesions of nearly 100 per cent the rate for the Europeans decreased to a considerable degree. This finding appears to indicate trends in opposite directions: an increase in caries prevalence and severity in the originally less afflicted oriental population, and a decrease in the children of occidental origin. Thus the gap between the different elements seems to narrow eventually, but considering the figures of the group "Israel" the findings appear to point to a trend of rising caries incidence. However, it should be borne in mind that a quarter of the "Israeli" live in kibbutzim, where caries experience is especially low, and on the other hand all the Yemenites found in this form of settlement are new immigrants. Caries experience with regard to type of residence is considered in another section, but the overlapping and interdependence of the various factors in any human society make it extremely difficult to isolate single features, let alone to establish causal relationships (FELDMAN, 1958).

CARIES EXPERIENCE IN RELATION TO TYPE OF RESIDENCE

An analysis of caries experience with regard to the form of settlement shows a statistically significant difference between kibbutz and village on one hand, and town on the other hand (Table 7). It is not justifiable to assume that this fact is due to the

TABLE 7. CARIES EXPERIENCE ACCORDING TO TYPE OF SETTLEMENT

Type of settlement	Community	D	M	F	DMF
Town	Jews	1·47	0·08	1·38	2·93
Town	Arabs	2·08	0·01	0·00	2·09
Village	Jews	1·82	0·08	0·35	2·25
Village	Arabs	2·23	0·07	0·00	2·30
Kibbutz	Jews	1·26	0·04	0·86	2·16

same causes as are responsible for the better dental health of rural populations reported elsewhere (BARNARD, 1956; HEWAT, 1956; TOVERUD, 1956–57), and summarized by LAMMERS and HAFER (1956). The rural Jewish settlements—collective as well as smallholders—are quite recent, most of them in fact were founded after the establishment of the State (STATISTICAL ABSTRACT, 1955/56). Therefore there

exists no old-established rural population, clinging conservatively to its traditional
way of living, in contrast to the city dwellers whose teeth deteriorate under the
influence of modern civilization and its food habits. Moreover, such important
commodities as sugar, meat and fat are rationed in Israel for all parts of the popula-
tion alike (rationing was abolished in the beginning of 1959), and considering the
small size of the country there is no difficulty in sending fresh agricultural produce
to the cities, and on the other hand processed and preserved food to the villages.
The better dental health in the villages in Israel is probably due to the fact that most
of the settlers are new immigrants belonging to the oriental communities; the situation
in the kibbutzim has already been discussed. There is no difference in the dental
health between the Arab rural and urban children.

It has been suggested (KANTOROWICZ, 1958) that the low caries experience in
kibbutzim might be due to exceptionally efficient infant care, which has practically
eradicated rickets in these settlements. Regardless of the controversy concerning
the role of vitamin D deficiency in the aetiology of dental caries it has been found
that rickets is prevalent in Israel to a surprisingly high extent, even in rural com-
munities (THAUSTEIN, 1959), and children are generally not sufficiently exposed to
sunshine—which would be possible about 300 days during the year—for a variety
of reasons. A vitamin A + D preparation is therefore uniformly distributed in the
whole country free of charge and mothers are advised to supplement the food of
children from the end of the first month throughout the second year of life, not-
withstanding the fact that breast feeding is generally practiced during a more or less
prolonged period in all parts of the population. Under these circumstances it is
difficult to see why rickets should be responsible for the higher caries experience of
urban children who receive the same antirachitic prophylaxis as their counterparts
in kibbutzim and villages. Rather it may be assumed that this difference is due
to the fact that children in kibbutzim are predominantly second generation born
in the country to immigrants from Europe, who are to a remarkable degree free from
caries. Considering the total population of Israel-born children this is more than
balanced by the higher caries prevalence of the second-generation Israelis of oriental
origin. In order to clarify this point it would be preferable to compare groups of
children indigenous in the country with newcomers of the same ethnic origin who have
not been reared in Israel and arrived only recently, for children who arrived at an
age of about 6 years in Israel and who have been included in the group of "old
established settlers" possibly conceal existing trends.

EXTENT OF TREATMENT TO CARIOUS LESIONS

There are several ways of assessing the degree to which needs resulting from
dental caries are being met. The "tooth mortality rate" (HAGAN, 1947) is based on
the factor "M" and indicates the number of extracted teeth per 100 individuals,
including those teeth whose extraction is indicated. Since this decision depends
largely on personal judgment it is a potential source of unpredictable bias; therefore
in the present survey all carious teeth still present in the mouth were included in
"D". Another method of assessing the degree to which needs are met by treatment

is to calculate the ratio between carious teeth restored and the total caries experience per 100 children (WISAN, 1957). The findings (Table 8) roughly reflect the cultural rather than the socio-economic level of the ethnic groups, though availability of treatment facilities from a geographical point of view is a factor of the utmost importance (MOEN, 1955), which is probably mainly responsible for the most unsatisfactory situation of the Arabs.

TABLE 8. PERCENTAGE OF RESTORATIVE NEEDS BEING MET (F/DMF): BY ORGIN

Origin	D	M	F	DMF	F/DMF × 100 (%)
Israel	1·60	0·05	1·30	2·95	44
Iraq–Iran	1·74	0·07	0·54	2·35	23
Yemen	1·25	0·04	0·74	2·03	36
Europe	1·39	0·07	1·39	2·85	49
Near East	1·72	0·01	1·24	2·97	42
North Africa	2·07	0·16	0·15	2·38	6
Arabs	2·21	0·06	0·00	2·26	0

DENTAL CARE WITH REGARD TO TYPE OF RESIDENCE

An entirely different grouping appears when the degree to which restorative needs are being met is considered. In the Jewish sector approximately half of the decayed teeth are treated in cities and kibbutsim, in villages only 11 per cent (Table 9).

These findings reflect the geographical distribution of dentists in Israel, three-quarters of whom live in urban communities, most of them in the three major cities (ROSENZWEIG, 1958). Kibbutzim, owing to their higher standard of living, generally conclude agreements with urban dentists, who visit them regularly in order to treat their members.

TABLE 9. PERCENTAGE OF RESTORATIVE NEEDS BEING MET: BY TYPE OF SETTLEMENT

Type of settlement	Community	F/DMF × 100 (%)
Town	Jews	46
Town	Arabs	0
Village	Jews	11
Village	Arabs	0
Kibbutz	Jews	40

RELATION BETWEEN CARIES PREVALENCE AND CARIES EXPERIENCE

The observed values of the mean DMF and the percentage of children with at least one DMF tooth were compared to KNUTSON's (1944) curve and equation $97 - y = 97(0.524)^x$, by which a relation is established between these variables. By substituting x for the observed values of the different groups, the expected values of

y were found, which differed significantly from those observed (Table 10). Knutson's curve was based on observations of American children. It appears that in all of the groups surveyed in Israel the pattern of caries prevalence differs significantly from the corresponding ones in U.S.A.: the age specific mean DMF is much lower, and the percentage of children without caries experience is much higher. Furthermore, the observed average DMF values were plotted against the corresponding percentages of children with at least one DMF tooth. An almost perfect straight line relation appeared, as indicated by the high correlation coefficient. Since in this section Knutson's curve may be considered as a straight line, it was decided to test the fit of Knutson's curve to the data obtained in Israel. A highly significant difference was observed (PEARSON, 1930) ($P = 0.011$). It is impossible to decide, on the basis of the few present observations, whether the correlation between mean DMF and percentage of children with at least one DMF tooth forms, for the population of Israel, a curve of the same type as Knutson's, with perhaps different constants.

TABLE 10. RELATIONSHIP OF THE PERCENTAGE OF CHILDREN WITH ONE OR MORE DMF TEETH TO THE AVERAGE NUMBER OF DMF TEETH PER CHILD, AND THE EXPECTED AVERAGE (KNUTSON)

Origin	Percentage with at least DMF = 1	Observed average	Expected average	Difference	S.E.[a]	Critical Ratio
Yemen	56.7	2.03	1.36	0.67	0.017	3.6
Arabs	60.9	2.26	1.52	0.74	0.011	5.0
Iraq–Iran	65.4	2.35	1.73	0.62	0.012	4.0
North Africa	61.1	2.38	1.53	0.85	0.012	5.4
Europe	73.4	2.85	2.18	0.67	0.004	7.3
Israel	73.5	2.95	2.18	0.77	0.043	2.6
Near East	78.6	2.97	2.57	0.40	0.042	1.3

The asymptote representing the upper limit of persons with caries experience may be lower than 97 per cent, or this limit may be reached at a lower rate of increase of the average DMF; in the latter case a different type of curve would result. It appears that caries experience, as expressed by average DMFT, is relatively higher in Israel than would have been expected on the basis of the formula which was calculated by Knutson for American children.

FLUOROSIS

In the whole sample of about 4500 children only fifty-four cases with evidence of fluorosis were found—about 1.2 per cent. Over four-fifths of these cases were immigrants, and among them the few who were graded as severe, and almost all the moderate cases. The highest prevalence of fluorosis was found in immigrants from Yemen, North Africa being second and Iraq third in order of frequency (Table 11). These findings indicate that at least in the aforementioned countries Jews lived in areas with endemic fluorosis, a fact which possibly has its bearing on the low prevalence of caries in the immigrants' sections of these groups.

TABLE 11. PREVALENCE OF FLUOROSIS

Group		Degree of fluorosis		
		Mild	Moderate	Severe
1. Israel		2	1	—
2. Iraq	(a) old-established	—	—	—
	(b) immigrants	4	4	—
3. N. Africa	(a) old-established	—	—	—
	(b) immigrants	9	3	—
4. Yemen	(a) old-established	1	—	—
	(b) immigrants	12	3	2
5. Europe	(a) old-established	1	—	—
	(b) immigrants	1	2	—
6. N. East	(a) old-established	1	—	—
	(b) immigrants	1	3	—
7. Arabs		4	—	—
Total	(a) old-established*	9	1	—
	(b) immigrants	27	15	2

* Including Arabs and Israel.

The amount of fluorosis found in the old established population is negligible, and bearing in mind the amount of fluorine in the local water supplies, according to the earlier quoted surveys, fluorosis is bound to disappear in Israel, except for immigrants from endemic areas, and certainly is no public health problem.

work of Drs. I. YANNAI and G. BEN-JOSSEF who put their knowledge, devotion and skill into the difficult task of carrying out the examinations in the field, even under the most adverse conditions.

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THE GRADIENT OF MINERALIZATION IN DEVELOPING ENAMEL

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Abstract—The gradient of mineralization in developing enamel of human deciduous teeth was assessed by measurements of intrinsic birefringence and the degree of mineralization expressed as a percentage of the intrinsic birefringence of fully formed enamel. The percentage of accessible spaces present in developing enamel was estimated from assessments of form birefringence after imbibition of the specimens in watery media of differing refractive indices. The gradient of mineralization as assessed by this method followed the general pattern demonstrated by microradiography. The percentage of imbibable spaces was smallest at the amelodentinal junction and at the enamel surface and greatest in the centre of the enamel.

INTRODUCTION

It has been previously demonstrated, by the use of microradiography (Crabb and Darling, 1958; Crabb, 1959) that human enamel mineralizes in two stages: (a) a primary phase in which the enamel matrix is deposited in increments delineated by the striae of Retzius and is then rapidly partially mineralized, and (b) a secondary phase in which a highly mineralized zone is laid down along the amelodentinal junction and then spreads out towards the enamel surface, advancing first in the region of the cusps. This latter phase has its advancing front at first parallel to the amelodentinal junction and later, as the enamel surface is approached, parallel to the outer border of the enamel. The secondary phase occurs shortly after the commencement of the primary phase so that the two phases occur concurrently throughout the greater part of the period of mineralization.

From the general pattern of mineralization seen in microradiographs (Fig. 1) it would appear that there is a gradient in the amount of mineral salts present which decreases from the amelodentinal junction to the enamel surface. This has been demonstrated by Hammarlund-Essler (1958) in curves constructed from a photometric analysis of microradiographs of developing enamel. Because these curves exhibit a smooth continuity Hammarlund-Essler considers that it is not possible to recognize distinct stages in the mineralization of the enamel. However, these curves represent a linear survey and their continuity does not preclude the existence of two phases advancing in different directions. On the basis of the overall microradiographic picture we still think that there is justification for the division of the mineralization process into two stages. In addition, further stages may be recognized in an examination of the fine structure of the developing enamel which reveals a primary preferential mineralization of the prism core in the mineralization of prism structure (Crabb, 1959).

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The present investigation is concerned with the assessment of the overall gradient of mineralization across developing enamel from the amelodentinal junction to the enamel surface by means of the measurement of birefringence in media with suitable refractive indices. In view of the assessment of mineralization made by HAMMAR-LUND-ESSLER (1958), using photometric analysis of microradiographs, it is valuable to compare the results obtained by these differing methods of approach to the same problem.

MATERIAL AND METHODS

Two ground sections of developing deciduous upper central incisors, of which microradiographs had previously been made (Figs. 2 and 3), were chosen for measurements of the intrinsic birefringence of the developing enamel. In order to avoid interference with the entry of imbibition media into the specimens, the ground sections were prepared without the use of embedding material but otherwise the technique used was that described by DARLING and CRABB (1956). The sections were then examined by polarized light in a watery medium of refractive index 1·62 (Thoulet's solution: DARLING, 1956) and surveys of the intrinsic birefringence were made by measuring the retardation at a series of nine points across the enamel from the amelodentinal junction to the enamel surface. In order to determine form birefringence, the retardation was also measured at alternate points of the same series in media of refractive index 1·33 (distilled water), 1·41 and 1·47 (modifications of the Thoulet's solution).

The thickness of the sections was then estimated using a technique of reflection of a slit source of light from a mirror. The mirror was mounted on a tripod in such a way that the insertion of a tooth section under one leg of the tripod would produce a measureable deflection of the slit image. The deflections produced could then be interpolated on a curve derived from measurements of metal strips of known thickness. The reproducibility of the method of measurement of enamel thickness was assessed by taking ten separate sets of readings relating "thickness" to "deflection". This was done for each thickness of metal used and in each of the ten sets of measurements the thickness of a tooth section was also measured at the enamel surface and at the amelodentinal junction. The same section was used in each case and an attempt made to measure the same points on the section. On this basis it was found that the measurements of thickness of the tooth section had a coefficient of variation of ±7·5 per cent for readings at the amelodentinal junction and ±3·0 per cent for readings at the enamel surface. The greater variation in assessments made at the amelodentinal junction as compared with those at the enamel surface is attributed to the difficulty in accurately locating the boundary between enamel and dentine compared with the relative ease of locating the outer border of the enamel.

Accuracy in the measurement of birefringence is dependent upon the accuracy of measurement of thickness. While there are obvious limitations to the method of measurement used as far as absolute values are concerned, the method adequately established whether or not a gradient of thickness existed across the enamel by

determining the difference in thickness between the enamel surface and the amelo-
dentinal junction. This allowed a more accurate estimate of the gradient of bire-
fringence than could have been obtained by using cruder measurements of section
thickness. Measurements of thickness of enamel in these surveys fall into the range
of 40–75 μ.

The intrinsic birefringence was then calculated from the retardation which had
been measured in the medium of refractive index 1·62 and from the thickness of the
section. The gradient of thickness across the enamel was taken into account when
present. Similarly the birefringence in media of refractive index of 1·33, 1·41 and
1·47 was also calculated for alternate points across the enamel. With this information
the total form birefringence which occurred in each medium could be assessed from
the difference between the intrinsic and the observed birefringence for that medium.
Because of the existence of high form birefringence in developing enamel it may be
implied that there is an organized distribution of "spaces" in the enamel and that this
distribution is not wholly random in nature. It should be mentioned that the term
"spaces" refers to sites where the exchange of imbibing media can occur with result-
ing form birefringence. It is probable that in developing enamel the spaces which
are present at any stage of mineralization are later occupied by crystallites which are
themselves known to be arranged in preferential orientation. Therefore it may be
expected that the spaces will be arranged in a preferentially oriented manner. The
percentage of "spaces" present in the developing enamel was therefore estimated
using the formula modified from Wiener (DARLING, 1958) relating "spaces" and
form birefringence. It is possible that some of these spaces may be oriented at angles
different from the usual parallel arrangement so that the resultant form birefringence
may indicate fewer spaces than actually exist at the point examined. The calculation
of spaces from form birefringence will therefore represent the minimum amount of
space which must be present.

In order to express "mineralization" as a percentage of mature enamel it was
necessary to examine the intrinsic birefringence of fully formed deciduous and
permanent teeth. Ten deciduous and five permanent teeth were examined and
measurements of birefringence were made at five points across the enamel from the
amelodentinal junction to the enamel surface.

With the information obtained about the birefringence of the developing and
the fully formed enamel an assessment was made of the degree of mineralization of
the developing enamel. "Mineralization" has been expressed as a percentage of the
mineralization of mature enamel according to the following formula,

$$\frac{\text{Intrinsic birefringence of developing enamel} \times 100}{\text{Intrinsic birefringence of fully formed enamel}}.$$

This has been done for each point across the enamel, giving an estimate of the gradient
of mineralization in relation to the mineralization of the fully formed deciduous
tooth. The mean values of birefringence for the five points across the enamel of the
fully formed deciduous tooth were used as a base line to which figures for the develop-
ing enamel could be related. In the detailed surveys of the two developing teeth

nine points across the enamel had been examined, whereas in the fully formed teeth only five points across the enamel had been assessed in each case. Therefore, in relating values for the nine points across the developing enamel to the findings for the five points in the fully formed enamel the latter were adjusted to correspond with points in the developing enamel. The intermediate values for mature enamel were calculated as a mean of the values on either side, so that a direct comparison could be made.
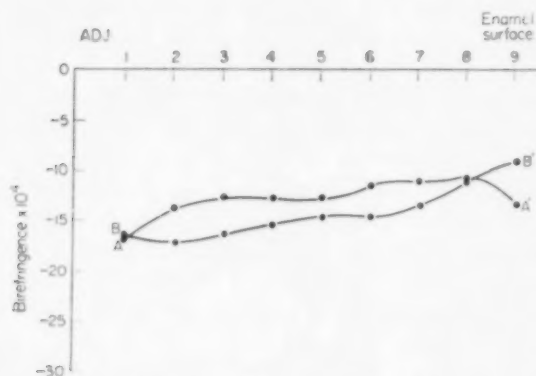
FIG. 4. Graph relating "intrinsic birefringence $\times 10^4$" to nine points across the enamel from the amelodentinal junction (ADJ) to the enamel surface for the labial (A–A') and incisal (B–B') enamel of the mineralizing deciduous incisor shown in Fig. 2 (Survey No. 1).
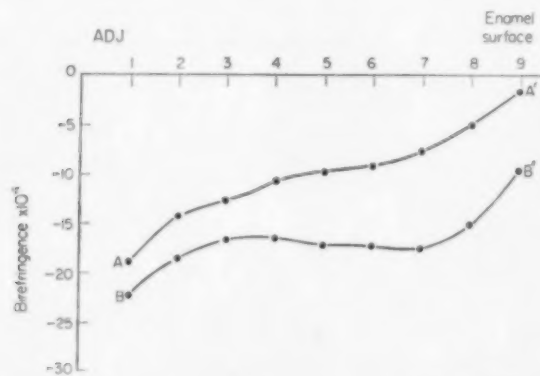
FIG. 5. Graph relating "intrinsic birefringence $\times 10^4$" to nine points across the enamel from the amelodentinal junction (ADJ) to the enamel surface for the labial A–A' and lingual B–B' enamel of the mineralizing deciduous central incisor shown in Fig. 3 (Survey No. 2).

RESULTS

The measurements of intrinsic birefringence which were obtained indicate (Figs. 4 and 5) that a gradient of birefringence exists in the developing enamel diminishing from the amelodentinal junction to the enamel surface. In each of the surveys the

birefringence at the amelodentinal junction was similar for labial, lingual and incisal enamel, within small limits, indicating that the degree of mineralization was of the same order. In the specimens examined the birefringence falls off towards the enamel surface except in the surface area of A–A' in survey No. 1 where there is a rise in birefringence at the enamel surface relative to the underlying enamel. This corresponds to the relative increase in radiopacity in the microradiograph in the same area, and is similar to the results of the microphotometric analysis of microradiographs by HAMMARLUND-ESSLER (1958). In survey No. 2 there is a sharp decrease in birefringence near the enamel surface. This may be because the tooth is at an earlier stage of development. The birefringence of fully formed teeth is shown in Tables 1 and 2 and the figures for the estimation of percentage "mineralization" and of "spaces" present in the developing enamel are shown in Tables 3 and 4. The birefringence of the enamel of fully formed permanent teeth appeared to be somewhat higher than that of the fully formed deciduous teeth, especially in the outer half of the enamel, but this is beyond the scope of the present investigation. For purposes of comparison only the measurements of the deciduous teeth were used so that the developing deciduous enamel was related to comparable material.

In terms of fully formed enamel the percentage "mineralization" (Tables 3 and 4) of the developing incisors fell in the range of 7–100 per cent. In view of the fact that the lingual enamel of incisors mineralizes in advance of the labial enamel (CRABB and DARLING, 1958; CRABB, 1959; ALLAN, 1959a,b) it is of interest that there is hardly any difference between the mineralization at the amelodentinal junction of the labial and lingual or incisal enamel. The range of "mineralization" at the

TABLE 1. MEASUREMENTS OF INTRINSIC BIREFRINGENCE OF FULLY FORMED DECIDUOUS ENAMEL

Five points from the amelodentinal junction to the enamel surface were measured in each tooth in a medium of refractive index 1·62 (Thoulet's solution). All measurements are of negative birefringence and are expressed as path difference/mμ × 10^4 (X = no reading obtained).

Tooth	ADJ	Point		Enamel surface	
	1	2	3	4	5
A/	19·1	21·3	24·4	25·1	24·4
A/	19·4	22·6	23·6	25·0	X
A/	18·4	21·0	24·2	25·0	16·8
/A	16·1	17·8	19·2	25·7	22·8
/A	14·9	16·4	19·3	19·6	12·4
/A	20·7	21·7	24·1	24·2	16·8
/B	22·5	24·5	24·3	24·6	23·2
/C	14·6	19·4	20·6	20·1	20·3
/D	15·0	21·4	18·8	23·6	20·5
/E	17·3	23·0	23·7	27·0	23·7
Mean	17·8	20·9	22·2	24·0	20·1
Standard deviation	±2·7	±2·4	±2·4	±2·4	±4·0

TABLE 2. MEASUREMENTS OF INTRINSIC BIREFRINGENCE OF FULLY FORMED PERMANENT ENAMEL

Five points from the amelodentinal junction to the enamel surface were measured in each tooth in a medium of refractive index 1·62 (Thoulet's solution). All measurements are of negative birefringence and are expressed as path difference/mμ × 10⁴

| Tooth | ADJ | Point | | | Enamel surface |
	1	2	3	4	5
/3	16·9	27·6	33·2	38·6	30·4
/4	17·6	17·9	20·0	24·7	25·7
/4	18·4	28·0	28·5	27·9	23·6
5/	17·4	24·8	32·2	32·1	27·4
/6	15·2	16·1	23·8	26·0	24·6
Mean	17·1	22·9	27·5	29·9	26·3
Standard deviation	±1·2	±5·6	±5·2	±5·5	±2·6

TABLE 3. MINERALIZATION OF DEVELOPING ENAMEL (SURVEY NO. 1) EXPRESSED AS A PERCENTAGE OF FULLY FORMED ENAMEL IN TERMS OF BIREFRINGENCE AND THE ESTIMATION OF PERCENTAGE "SPACES" PRESENT IN THE DEVELOPING ENAMEL FROM THE FORM BIREFRINGENCE ASSESSED IN VARIOUS MEDIA

Form birefringence for points 1, 3, 5, 7 and 9 was measured and for points 2, 4, 6 and 8 was estimated from curves based on observed birefringence in the various media used

| Labial enamel A–A' | ADJ | | Point | | | | | Enamel surface | |
	1	2	3	4	5	6	7	8	9
% "Mineralization"	94	71	61	60	59	49	47	48	67
% "Spaces" assessed in media of refractive index 1·47	10	13	14	16	16	16	14	13	12
1·41	8	12	18	20	20	20	20	18	17
1·33	11	13	16	17	18	18	18	16	14
Average % "spaces"	10	13	16	18	18	18	17	16	14

| Incisal enamel B–B' | ADJ | | Point | | | | | Enamel surface | |
	1	2	3	4	5	6	7	8	9
% "Mineralization"	93	89	78	72	66	64	56	51	46
% "Spaces" assessed in media of refractive index 1·47	1	4	6	7	10	10	9	8	7
1·41	1	3	5	7	10	10	9	9	7
1·33	1	3	5	7	9	8	8	6	6
Average % "spaces"	1	3	5	7	10	9	9	8	7

TABLE 4. MINERALIZATION OF DEVELOPING ENAMEL (SURVEY NO. 2) EXPRESSED AS A PERCENTAGE OF FULLY FORMED ENAMEL IN TERMS OF BIREFRINGENCE AND THE ESTIMATION OF PERCENTAGE "SPACES" PRESENT IN THE DEVELOPING ENAMEL FROM THE FORM BIREFRINGENCE ASSESSED IN VARIOUS MEDIA

Form birefringence for points 1, 3, 5, 7 and 9 was measured and for points 2, 4, 6 and 8 was estimated from curves based on observed birefringence in the various media used

Labial enamel A–A′	ADJ		Point					Enamel surface	
	1	2	3	4	5	6	7	8	9
% "Mineralization"	100	73	60	49	43	39	32	22	7
% "Spaces" assessed in media									
of refractive index 1·47	9	13	16	19	20	22	21	19	13
1·41	10	15	20	23	25	28	27	24	18
1·33	10	13	16	20	23	25	24	21	17
Average % "spaces"	10	14	17	21	23	25	24	21	16

Lingual enamel B–B′	ADJ		Point					Enamel surface	
	1	2	3	4	5	6	7	8	9
% "Mineralization"	100	95	79	75	76	73	71	69	47
% "Spaces" assessed in media									
of refractive index 1·47	8	9	12	12	14	17	19	18	12
1·41	5	8	11	13	14	15	17	17	13
1·33	3	6	9	12	14	16	17	16	12
Average % "spaces"	5	8	11	12	14	16	18	17	12

amelodentinal junction of the developing incisors is from 93 to 100 per cent. The outer border of the enamel shows greater variation in "mineralization", ranging from 7 to 67 per cent. The outer border of the enamel, during part of the time of mineralization, is more mineralized than the immediately underlying enamel (HAMMARLUND-ESSLER, 1958; CRABB, 1959) and may be undergoing a distinctly separate phase of mineralization. It would be expected that the degree of mineralization recorded would vary considerably with the stage of development and with the particular part of the surface examined. The percentage of "spaces" as assessed from form birefringence is small (1–10 per cent) at the amelodentinal junction and also at the enamel surface (7–16 per cent), whereas the percentage of spaces in the centre of the enamel is greater, ranging from 10 to 25 per cent.

DISCUSSION

It can be seen that the figures for birefringence of fully formed deciduous enamel (Table 1) are somewhat lower than those for permanent enamel (Table 2). Previous figures given in the literature for the birefringence of fully formed enamel expressed

as "birefringence × 10^4" are as follows:

VON EBNER (1906)	43
CAPE and KITCHIN (1930)	30
KEIL (1936)	29
HARDERS-STEINHAUSER (1938)	25
DARLING (1956)	30–40

Most of these figures are higher than those given in Table 1 for deciduous enamel but are comparable to the figures for permanent enamel in Table 2. The lowest figure in the literature, given by HARDERS-STEINHAUSER, is 25 and was in fact based on calculations from measurements obtained from developing deciduous enamel. In the case of DARLING's figures the values relate to the outer part of the enamel of permanent teeth and are comparable to the figures for the outer part of the enamel given in Table 2. It appears therefore that there may be a difference in the intrinsic birefringence of deciduous and permanent enamel and for purposes of the present investigation only the figures for the deciduous enamel (Table 1) were used as a basis of assessment.

The gradients of birefringence and the assessments of mineralization in the developing enamel confirm the general qualitative pattern demonstrated by micro-radiography. The pattern of "spaces" as assessed from form birefringence is as much as 25 per cent in the centre of the enamel and decreases towards the amelodentinal junction and the enamel surface (Tables 3 and 4). Some explanation is necessary to account for the existence of "spaces" in developing enamel and it is reasonable to suppose that they provide room for the deposition of the mineral salts. If the amount of organic matrix was constant it might be expected that the percentages of spaces would be directly complementary to the percentage mineralization. However, this is not so, except perhaps where the enamel is almost fully mineralized. which implies that these spaces are probably available for the final mineral deposition. The work of DEAKINS (1942) and WEINMANN, WESSINGER and REED (1942) indicates that organic matrix is withdrawn during the mineralization process. The approximately comple-mentary relationship between "spaces" and "mineralization" in the almost fully mineralized enamel near the amelodentinal junction (Tables 3 and 4) suggests that the organic matrix is removed just in advance of the mineral deposition, thus providing the space required for the crystallites. If this is so then the increase of spaces towards the centre of the enamel suggests that this is the zone of greatest mineralizing activity in the specimens examined. Nearer the amelodentinal junction the process is being completed while in the less mineralized parts of the enamel nearer the surface, where the available spaces are also less, the withdrawal of organic matrix may be considered to be less advanced.

There is, however, an apparent discrepancy in the relationship between assessed "mineralization" and "spaces" at the amelodentinal junction, at all such points examined in both specimens. Whereas "mineralization" in survey No. 2 (Table 4), for example, is estimated for both lingual and labial enamel at 100 per cent, the percentage of "spaces" for lingual and labial enamel is estimated at 5 and 10 per cent respectively. The discrepancy between the two sets of figures may be attributable to

the experimental error in combining the two methods of assessment. The experimental error could be accounted for by the variation in the measurements of birefringence of the fully formed teeth. The standard deviation for these measurements varied from 10 to 20 per cent of the mean reading for each point across the enamel. On the other hand, since "mineralization" has been calculated as a percentage of the mineralization of the fully formed enamel, any factors altering the birefringence of the fully formed enamel would affect the assessment of mineralization of the developing enamel. For example, incomplete mineralization of the fully formed enamel could have this effect. In this connection it is of some interest that HAMMARLUND-ESSLER (1958) gives a figure of 83 per cent mineral content by volume for the enamel of the erupted deciduous tooth. This figure is based on a quantitative assessment of microradiographs by photometric analysis and is much lower than one might expect. It is relevant therefore to consider what the proportions of organic material and moisture may be in the enamel of the erupted deciduous tooth. BURNETT and ZENEWITZ (1958) find the moisture content of the enamel to be $2 \cdot 2 \pm 0 \cdot 4$ per cent by weight. BIRD et al. (1940) find the moisture content of the enamel of permanent teeth to be $2 \cdot 3$ per cent by weight and of deciduous teeth to be $2 \cdot 8$ per cent by weight. STACK (1953 and personal communication) finds the organic content of deciduous enamel to be $0 \cdot 7$ per cent by weight, and that there is a proportional increase in ratio of water to organic material as enamel mineralizes, reaching a ratio of approximately 3 to 1 in the almost fully mineralized enamel. BIRD et al., however, give the extremely high figure of $4 \cdot 7$ per cent by weight for the organic content of the enamel of deciduous teeth. The figures for moisture content of enamel given by BIRD et al. and by BURNETT and ZENEWITZ are in agreement for permanent teeth, the figure for deciduous teeth being slightly higher and closely related to the figure given by STACK (1953) for organic content in approximately the ratio determined by STACK (personal communication) of 3 to 1. It is therefore proposed to assume the figure given by STACK (1953) for organic content rather than that of BIRD et al. and to assume the figure of $2 \cdot 8$ per cent given by the latter workers for the moisture content of deciduous enamel as approximations. In terms of percentage by volume these figures would become $1 \cdot 5$ per cent organic material, $7 \cdot 9$ per cent moisture with $90 \cdot 6$ per cent mineral content remaining. Now, if 83 per cent were the total mineral content of the enamel of the erupted deciduous tooth, as HAMMARLUND-ESSLER (1958) finds, then the percentage of organic material and moisture would need to be considerably higher; the exact ratio of moisture to organic material by weight would probably be of the order of 3 to 1 or less. HAMMARLUND-ESSLER (personal communication) considers that the remaining 17 per cent is probably mostly water and is at present investigating this problem further.

The figures for the zone of greatest mineralization of the enamel of the developing incisors (Tables 3 and 4) although related to fully formed enamel, are rather surprisingly higher than the figure (83 per cent) given by HAMMARLUND-ESSLER (1958) for the degree of mineralization of the enamel of erupted teeth. The possibility of experimental error has been already referred to. An alternative explanation may lie in the fact that the birefringence of the fully formed deciduous enamel, apart

from the enamel near the amelodentinal junction, is low compared with permanent enamel. A low birefringence for fully formed enamel would contribute a higher figure for "mineralization" of developing enamel expressed in terms of relative birefringence. A relatively lower birefringence in the enamel of the erupted deciduous tooth might be related to incomplete mineralization of the enamel as HAMMARLUND-ESSLER's findings would suggest. In this connexion it is of interest that BIRD *et al.* found a higher moisture content of deciduous enamel compared with permanent enamel. If one accepts the possibility that the moisture was occupying inaccessible spaces of the sort described by KEIL (1936) and that these spaces were aligned parallel to the crystallite axis, then the observed negative birefringence might be reduced. The figure given by BURNETT and ZENEWITZ (1958) of $2 \cdot 2 \pm 0 \cdot 4$ per cent for the moisture content of enamel was achieved by dehydration at 197°C in vacuo, but at 61°C the figure was only $1 \cdot 7 \pm 0 \cdot 7$ per cent. This would lend support to the idea that at room temperature moisture may be retained in the relatively inaccessible spaces which KEIL (1936) found to be present on heating enamel to 150°C.

While HAMMARLUND-ESSLER (1958) and ourselves have arrived at our conclusions by different techniques there is a considerable measure of agreement about the degree of mineralization of the enamel of developing deciduous incisors at birth. The figure given by HAMMARLUND-ESSLER for the mineral content of the most mineralized part of a developing deciduous incisor from a full term foetus was 78 per cent. If this figure is expressed as a percentage of her figure for fully formed enamel (83 per cent) it becomes 94 per cent, which is comparable to our own figure of 93–100 per cent, in which "mineralization" has also been expressed as a percentage of the fully formed enamel, but on a basis of birefringence.

The findings of HAMMARLUND-ESSLER (1958), that a gradient of mineralization exists in the developing tooth from the amelodentinal junction to the enamel surface, are in general agreement with the present findings. Also, as mineralization proceeds the external layer of enamel reaches at one stage a higher degree of mineralization than the immediately underlying enamel.

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FIG. 1. Microradiograph of a ground section of an upper left deciduous first molar from an infant of 22 weeks. The variation in radiopacity from the amelodentinal junction to the enamel surface is demonstrated. ×9.

FIG. 2. Microradiograph of the incisal tip of a ground section of an upper deciduous central incisor from an infant of 4 weeks. (Birth was near full term). A–A' and B–B' represent the path of the surveys made to determine the birefringence (Survey No. 1). ×37.

FIG. 3. Microradiograph of the incisal tip of a ground section of an upper central deciduous incisor from an infant of less than 1 week. (Birth was near full term). A–A' and B–B' represent the path of the surveys made to determine the birefringence (Survey No. 2). ×37.

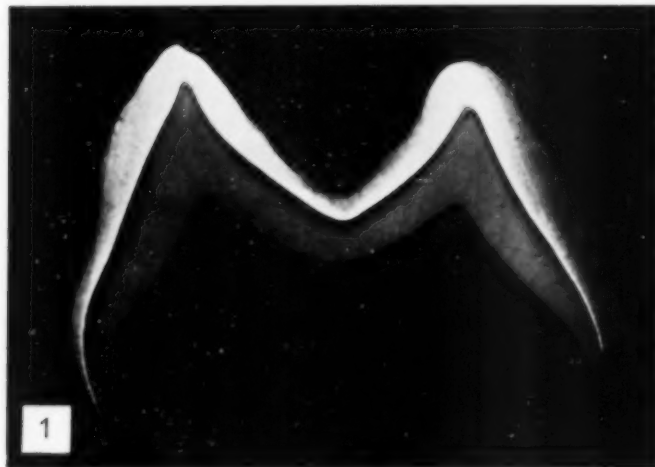PLATE 1

SHORT COMMUNICATION

THE CITRIC ACID CONTENT OF OX BONE

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IT HAS recently been reported (HARTLES and LEAVER, 1960; LEAVER, EASTOE and HARTLES, 1960; TAYLOR, 1960) that dentine contains citric acid linked with a peptide and that ethylenediamine tetra-acetic acid (EDTA) used to remove the mineral of dentine also releases the total citric acid component. At the time these papers were published we had just observed that the quantity of citric acid liberated from bone by EDTA was much greater than the amount released by trichloracetic acid which, since the early work of DICKENS (1941), is generally used for citric acid determination in both hard and soft tissues.

The results we have obtained on bone may be summarized as follows: citric acid of defatted and dried total ox bone was extracted with trichloracetic acid and measured colorimetrically by the pentabromacetone method. In a sample of adult diaphyseal bone 470 μg per 100 mg was found. No greater quantity was extracted if the extraction time (minimum period 24 hr) or temperature were increased, and the amount extracted was not influenced by the concentration of trichloracetic acid used. If a second extraction with the same reagent was made, citric acid was not found in this liquid phase.

If EDTA was used instead of trichloracetic acid, the liquid phase contained a much greater amount of citric acid, for example 900 μg per 100 mg in the afore-mentioned sample of bone.

If the solid phase remaining after trichloracetic acid extraction was treated with EDTA it was found that it still contained as much as 200 μg of citric acid per 100 mg which had escaped extraction with trichloracetic acid.

If a trichloracetic acid extract was treated with EDTA before applying the penta-bromacetone method an additional amount of citric acid appears (600 μg per 100 mg instead of 470 μg).

It may therefore be concluded that EDTA extracts from bone a fraction of citric acid which is not extracted by treatment with trichloracetic acid. Furthermore, part of the citric acid extracted by trichloracetic acid exists in a form not detectable as citric acid by the pentabromacetone method unless pretreated with EDTA.

In accord with the view of LEAVER et al. (1960) in respect of dentine, we believe that a citric acid complex is liberated by acids and dissociated by EDTA. Moreover, we believe that other forms of citric acid, which are dissociated only by EDTA, are present in calcified tissues.

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NOTICE

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The American Institute of Biological Sciences is currently translating and publishing seven Russian research journals in biology. These journals are translated with support from the National Science Foundation, which is eager that such information be more widely distributed to biologists throughout the world. It is hoped that this material will aid biologists in research, prevent duplication of work, give some idea of the work being done by Soviet scientists in the field of biology, and also bring about a better international understanding among scientists.

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The journals currently being translated are: *Doklady: Biological Sciences Section; Doklady: Botanical Sciences Section; Doklady: Biochemistry Section; Plan¹ Physiology; Microbiology; Soviet Soil Science;* and *Entomological Review.*

In addition to its programme of Russian Biological Journal translations, the AIBS has instituted a separate programme of translation and publication of selected Russian Monographs in biology.

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